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<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top; border-right: 1px solid black; padding-right: 10px;"> <p>(21) International Application Number: PCT/CA95/00557</p> <p>(22) International Filing Date: 2 October 1995 (02.10.95)</p> <p>(30) Priority Data: 08/316,407 30 September 1994 (30.09.94) US</p> <p>(71) Applicant: THE UNIVERSITY OF BRITISH COLUMBIA (CA/CA); IRC 331, 2194 Health Sciences Mall, Vancouver, British Columbia V6T 1Z3 (CA).</p> <p>(72) Inventors: HOLLAND, John, W.; 249 Bridge Road, Glebe, NSW 2039 (AU). MADDEN, Thomas, D.; 2714 West 31st Avenue, Vancouver, British Columbia V6L 2A1 (CA). CULLIS, Pieter, R.; 3732 West 1st Street, Vancouver, British Columbia V6R 1H4 (CA).</p> <p>(74) Agents: CLARK, Geoffrey, C. et al.; Fetherstonhaugh & Co., 1010 - 510 Burrard Street, Vancouver, British Columbia V6C 3A8 (CA).</p> </td> <td style="width: 50%; vertical-align: top; padding-left: 10px;"> <p>(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).</p> <p>Published <i>With international search report.</i></p> </td> </tr> </table>			<p>(21) International Application Number: PCT/CA95/00557</p> <p>(22) International Filing Date: 2 October 1995 (02.10.95)</p> <p>(30) Priority Data: 08/316,407 30 September 1994 (30.09.94) US</p> <p>(71) Applicant: THE UNIVERSITY OF BRITISH COLUMBIA (CA/CA); IRC 331, 2194 Health Sciences Mall, Vancouver, British Columbia V6T 1Z3 (CA).</p> <p>(72) Inventors: HOLLAND, John, W.; 249 Bridge Road, Glebe, NSW 2039 (AU). MADDEN, Thomas, D.; 2714 West 31st Avenue, Vancouver, British Columbia V6L 2A1 (CA). CULLIS, Pieter, R.; 3732 West 1st Street, Vancouver, British Columbia V6R 1H4 (CA).</p> <p>(74) Agents: CLARK, Geoffrey, C. et al.; Fetherstonhaugh & Co., 1010 - 510 Burrard Street, Vancouver, British Columbia V6C 3A8 (CA).</p>	<p>(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).</p> <p>Published <i>With international search report.</i></p>
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<p>(54) Title: BILAYER STABILIZING COMPONENTS AND THEIR USE IN FORMING PROGRAMMABLE FUSOGENIC LIPOSOMES</p>				
<p>(57) Abstract</p> <p>The present invention provides a fusogenic liposome comprising a lipid capable of adopting a non-lamellar phase, yet capable of assuming a bilayer structure in the presence of a bilayer stabilizing component; and a bilayer stabilizing component reversibly associated with the lipid to stabilize the lipid in a bilayer structure. Such fusogenic liposomes are extremely advantageous because the rate at which they become fusogenic can be not only predetermined, but varied as required over a time scale ranging from minutes to days. Control of liposome fusion can be achieved by modulating the chemical stability and/or exchangeability of the bilayer stabilizing component(s). The fusogenic liposomes of the present invention can be used to deliver drugs, peptide, proteins, RNA, DNA or other bioactive molecules to the target cells of interest.</p>				

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5 **BILAYER STABILIZING COMPONENTS AND THEIR USE IN FORMING
PROGRAMMABLE FUSOGENIC LIPOSOMES**

BACKGROUND OF THE INVENTION

10 It is well recognized in the medical field that the most effective procedure for treating localized disease is to direct the pharmaceutical or drug agent (hereinafter "drugs") to the affected area, thereby avoiding undesirable toxic effects of systemic treatment. Techniques currently being used to deliver drugs to specific target sites within the body involve the utilization of time-release capsules or gel matrices from which drugs slowly "leak," or the use of implantable "syringes" that mechanically release drugs into muscles or into the blood stream. Another, and perhaps more effective delivery system, 15 encompasses the use of liposomes containing the appropriate drug or chemical. The liposome with encapsulated drug is directed to the specific area of interest and, thereafter, the drug is released. The carrying out of this latter step is the most problematic and, in fact, the greatest barrier to the use of liposomes as drug carriers is making the liposomes release the drugs on demand at the target site of interest.

20 Liposomes are vesicles comprised of one or more concentrically ordered lipid bilayers which encapsulate an aqueous phase. They are normally not leaky, but can become leaky if a hole or pore occurs in the membrane, if the membrane is dissolved or degrades, or if the membrane temperature is increased to the phase transition temperature, T_c . Current methods of drug delivery via liposomes require that the 25 liposome carrier will ultimately become permeable and release the encapsulated drug at the target site. This can be accomplished, for example, in a passive manner wherein the liposome bilayer degrades over time through the action of various agents in the body. Every liposome composition will have a characteristic half-life in the circulation or at other sites in the body and, thus, by controlling the half-life of the liposome composition, 30 the rate at which the bilayer degrades can be somewhat regulated.

In contrast to passive drug release, active drug release involves using an agent to induce a permeability change in the liposome vesicle. Liposome membranes can be constructed so that they become destabilized when the environment becomes acidic near the liposome membrane (*see, e.g., Proc. Natl. Acad. Sci. USA* 84:7851 (1987);

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Biochemistry 28:908 (1989)). When liposomes are endocytosed by a target cell, for example, they can be routed to acidic endosomes which will destabilize the liposome and result in drug release. Alternatively, the liposome membrane can be chemically modified such that an enzyme is placed as a coating on the membrane which slowly destabilizes the liposome. Since control of drug release depends on the concentration of enzyme initially placed in the membrane, there is no real effective way to modulate or alter drug release to achieve "on demand" drug delivery. The same problem exists for pH-sensitive liposomes in that as soon as the liposome vesicle comes into contact with a target cell, it will be engulfed and a drop in pH will lead to drug release.

In addition to the foregoing methods, a liposome having a predetermined phase transition temperature, T_c , above body temperature can be used to achieve active drug delivery. In this method, the body temperature will maintain the liposome below the T_c so that the liposome will not become leaky when placed in the body. This method of drug release is capable of "on demand" drug delivery since such liposomes experience a greatly increased membrane permeability at their T_c which, in turn, enables drug or chemical release. To release drugs from such phase transition liposomes when in the body, heat must be applied until the T_c is achieved. Unfortunately, the application of heat can, in itself, create problems within the body and, frequently, the adverse effects of the heat treatment outweigh the beneficial effects of using the liposome as a drug delivery vehicle. Moreover, such liposomes must be made of highly purified and expensive phase transition temperature phospholipid materials.

In view of the foregoing, there exists a need in the art for a method for targeted drug delivery that overcomes the disadvantages of the currently available methods. Specifically, a parenteral delivery system is required that would be stable in the circulation, following intravenous administration, allowing retention of encapsulated or associated drug or therapeutic agent(s). This delivery system would be capable of accumulating at a target organ, tissue or cell via either active targeting (*e.g.*, by incorporating an antibody or hormone on the surface of the liposomal vehicle) or via passive targeting, as seen for long-circulating liposomes. Following accumulation at the target site, the liposomal carrier would become fusogenic, without the need for any external stimulus, and would subsequently release any encapsulated or associated drug or therapeutic agent in the vicinity of the target cell, or fuse with the target cell plasma membrane introducing the drug or therapeutic agent into the cell cytoplasm. In certain

instances, fusion of the liposomal carrier with the plasma membrane would be preferred because this would provide more specific drug delivery and, hence, minimize any adverse effects on normal, healthy cells or tissues. In addition, in the case of therapeutic agents such as DNA, RNA, proteins, peptides, *etc.*, which are generally not permeable to the cell membrane, such a fusogenic carrier would provide a mechanism whereby the therapeutic agent could be delivered to its required intracellular site of action. Further, by avoiding the endocytic pathway, the therapeutic agent would not be exposed to acidic conditions and/or degradative enzymes that could inactivate said therapeutic agent. Quite surprisingly, the present invention addresses this need by providing such a method.

SUMMARY OF THE INVENTION

The present invention provides a fusogenic liposome comprising a lipid capable of adopting a non-lamellar phase, yet capable of assuming a bilayer structure in the presence of a bilayer stabilizing component; and a bilayer stabilizing component reversibly associated with the lipid to stabilize the lipid in a bilayer structure. Such fusogenic liposomes are extremely advantageous because the rate at which they become fusogenic can be not only predetermined, but varied as required over a time scale ranging from minutes to days. Control of liposome fusion can be achieved by modulating the chemical stability and/or exchangeability of the bilayer stabilizing component(s).

Lipids which can be used to form the fusogenic liposomes of the present invention are those which adopt a non-lamellar phase under physiological conditions or under specific physiological conditions, *e.g.*, in the presence of calcium ions, but which are capable of assuming a bilayer structure in the presence of a bilayer stabilizing component. Lipids which adopt a non-lamellar phase include, but are not limited to, phosphatidylethanolamines, ceramides, glycolipids, or mixtures thereof. Such lipids can be stabilized in a bilayer structure by bilayer stabilizing components which are either bilayer forming themselves, or which are of a complementary dynamic molecular shape. More particularly, the bilayer stabilizing components of the present invention must be capable of stabilizing the lipid in a bilayer structure, yet they must be capable of exchanging out of the liposome, or of being chemically modified by endogenous systems so that, with time, they lose their ability to stabilize the lipid in a bilayer structure, thereby allowing the liposome to become fusogenic. Only when liposomal stability is

lost or decreased can fusion of the liposome with the plasma membrane of the target cell occur.

By controlling the composition and concentration of the bilayer stabilizing component, one can control the chemical stability of the bilayer stabilizing component and/or the rate at which the bilayer stabilizing component exchanges out of the liposome and, in turn, the rate at which the liposome becomes fusogenic. In addition, other variables including, for example, Ph, temperature, ionic strength, etc. can be used to vary and/or control the rate at which the liposome becomes fusogenic.

The fusogenic liposomes of the present invention are ideally suited to a number of therapeutic, research and commercial applications. In therapeutic applications, for example, the initial stability of the fusogenic liposome would allow time for the liposome to achieve access to target organs or cells before attaining its fusogenic state, thereby reducing non-specific fusion immediately following administration.

In addition, the fusogenic liposomes of the present invention can be used to deliver drugs, peptide, proteins, RNA, DNA or other bioactive molecules to the target cells of interest. In this embodiment, the compound or molecule to be delivered to the target cell can be encapsulated in the aqueous interior of the fusogenic liposome and subsequently introduced into the cytoplasm (initially) upon fusion of the liposome with the cell plasma membrane. Alternatively, molecules or compounds can be embedded within the liposome bilayer and, in this case, they would be incorporated into the target cell plasma membrane upon fusion.

As such, in another embodiment, the present invention provides a method for delivering a therapeutic compound to a target cell at a predetermined rate, the method comprising: administering to a host containing the target cell a fusogenic liposome which comprises a bilayer stabilizing component, a lipid capable of adopting a non-lamellar phase, yet capable of assuming a bilayer structure in the presence of the bilayer stabilizing component, and a therapeutic compound or a pharmaceutically acceptable salt thereof. Administration may be by a variety of routes, but the therapeutic compounds are preferably given intravenously or parenterally. The fusogenic liposomes administered to the host may be unilamellar, having a mean diameter of 0.05 to 0.45 microns, more preferably from 0.05 to 0.2 microns.

In a final embodiment, the present provides a method of stabilizing in a bilayer structure a lipid which is capable of adopting a non-lamellar phase by combining

the lipid(s) with a bilayer stabilizing component. Once stabilized, the lipid mixture can be used to form the fusogenic liposomes of the present invention. The bilayer stabilizing component is selected, however, to be exchangeable such that upon loss of this component from the liposome, the liposome is destabilized and becomes fusogenic.

Other features, objects and advantages of the invention and its preferred embodiments will become apparent from the detailed description which follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the concentration dependence of bilayer stabilization by a bilayer stabilizing component (BSC). Multilamellar vesicles were prepared, as described in the examples, from mixtures of DOPE:cholesterol:DOPE-PEG₂₀₀₀, 1:1:N, where N is the proportion of DOPE-PEG₂₀₀₀ as indicated in the Figure 1. ³¹P-NMR spectra were determined at 20°C after the sample had been allowed to equilibrate for 30 minutes.

Figure 2 illustrates the temperature dependence of bilayer stabilization by BSC. Multilamellar vesicles were prepared, as described in the examples, from mixtures of DOPE:cholesterol:DOPE-PEG₂₀₀₀ at a ratio of: A, 1:1:0.1; or B, 1:1:0.25. The samples were cooled to 0°C and ³¹P-NMR spectra were determined from 0°C to 60°C at 10°C intervals. The samples were allowed to equilibrate at each temperature for 30 min. prior to data accumulation.

Figure 3 illustrates the effect of headgroup size on the bilayer stabilizing ability of BSC. Multilamellar vesicles were prepared from either A, DOPE:cholesterol:DOPE-PEG₂₀₀₀, 1:1:0.05, or B, DOPE:cholesterol:DOPE-PEG₅₀₀₀, 1:1:0.05. Other conditions were the same as for Figure 2.

Figure 4 illustrates the effect of the acyl chain composition on the bilayer stabilizing ability of BSC. Multilamellar vesicles were prepared, as described in the examples, from either A, DOPE:cholesterol:DMPE-PEG₂₀₀₀, 1:1:0.1, B, DOPE:cholesterol:DPPE-PEG₂₀₀₀, 1:1:0.1, or C, DOPE:cholesterol:DSPE-PEG₂₀₀₀, 1:1:0.1. Other conditions were the same as for Figure 2.

Figure 5 illustrates the ability of PEG-Ceramide to act as a bilayer stabilizing component. Multilamellar vesicles were prepared, as described in the examples, from DOPE:cholesterol:egg ceramide-PEG₂₀₀₀ at a ratio of A, 1:1:0.1 or B, 1:1:0.25. Other conditions were the same as for Figure 2.

Figure 6 illustrates the freeze-fracture electron micrograph of MLVs prepared from DOPE:cholesterol:DOPE-PEG₂₀₀₀ (1:1:0.1). The samples were prepared as described in the examples. The bar represents 500 nm.

Figure 7 illustrates the freeze-fracture electron micrograph of LUVs prepared from DOPE:cholesterol:DOPE-PEG₂₀₀₀ (1:1:0.1). The samples were prepared as described in the examples. The bar represents 500nm.

Figure 8 illustrates the elution profiles of LUVs prepared from DOPE:cholesterol:DSPE-PEG₂₀₀₀, and micelles composed of DSPE-PEG₂₀₀₀. LUVs were prepared, as described in the examples, from DOPE:cholesterol:DSPE-PEG₂₀₀₀ (1:1:0.1) with trace amounts of ¹⁴C-DPPC (Δ) and ³H-DSPE-PEG₂₀₀₀ (●). They were chromatographed as described in the examples. In a separate experiment, micelles were prepared from DSPE-PEG₂₀₀₀ labelled with ³H-DSPE-PEG₂₀₀₀ (○) and chromatographed on the same Sepharose 4B column.

Figure 9 illustrates the inhibition of fusion by PEG-PE. Liposomes were prepared from equimolar mixtures of DOPE and POPS containing (a) 0; (b) 0.5; (c) 1 or (d) 2 mol % DMPE-PEG₂₀₀₀. In addition to the above lipids, labelled liposomes also contained the fluorescent lipids NBD-PE and Rh-PE at 0.5 mol%. Fluorescently labelled liposomes (final concentration 60 μM) were incubated at 37°C for 30s before the addition of a three-fold excess of unlabelled liposomes, followed one minute later by CaCl₂ (final concentration 5 mM).

Figure 10 illustrates the recovery of fusogenic activity after PEG-PE removal. Fusion between fluorescently labelled and unlabelled liposomes containing 2 mol% DMPE-PEG₂₀₀₀ was assayed as described under Figure 9, except that one minute after addition of CaCl₂, a 12-fold excess (over labelled vesicles) of POPC liposomes (curve a) or an equivalent volume of buffer (curve b) was added.

Figure 11 illustrates the concentration dependence of recovery of fusogenic activity. Fusion between fluorescently labelled and unlabelled liposomes containing (a) 2; (b) 3; (c) 5 or (d) 10 mol% DMPE-PEG₂₀₀₀ was assayed as described under Figure 10, except that POPC liposomes were added as a 36-fold excess over labelled vesicles.

Figure 12 illustrates programmable fusion. Fusion between fluorescently labelled and unlabelled liposomes containing 2 mol% of the indicated PE-PEG₂₀₀₀ was assayed as described under Figure 10. The % fusion was calculated as described in the

examples. (A) DMPP-PEG₂₀₀₀ (●); DPPE-PEG₂₀₀₀ (◆); DSPE-PEG₂₀₀₀ (▲); and
(B) DOPE-PEG₂₀₀₀ (▲), egg ceramide-PEG₂₀₀₀ (▼).

Figure 13 illustrates the effect of PEG molecular weight on fusion.

(A) Assays were carried out as described in Figure 9 using liposomes which contained
(a) 0; (b) 0.25; (c) 0.5 or (d) 1 mol% DMPE-PEG₅₀₀₀; and (B) Assays were performed
as described under Figure 12 using liposomes which contained 1 mol% DMPE-PEG₅₀₀₀
(●); DPPE-PEG₅₀₀₀ (◆) or DSPE-PEG₅₀₀₀ (▲).

Figure 14 illustrates the comparison of PEG₂₀₀₀ to PEG₅₀₀₀ at equal
concentration of oxyethylene groups. Liposomes contained either 2 mol% PEG₅₀₀₀
(upper curve) or 5 mol% PEG₂₀₀₀ (lower curve). Other conditions were as described
under Figure 11.

Figure 15 illustrates the effect of salt concentration on fusion of
DOPE:DODAC Liposomes. Liposomes were prepared from DOPE:DODAC (85:15).
Donor liposomes also contained the fluorescent lipids, NBD-PE and Rh-PE at 0.5mol%.
Donor liposomes (final concentration 60μM) were incubated at 37°C for 30s before the
addition of a three-fold excess of unlabelled acceptor liposomes followed 1 min later by
NaCl to give the indicated final concentration.

Figure 16 illustrates the inhibition of fusion of DOPE:DODAC liposomes
by PEG-PE. Liposomes were prepared from either DOPE:DODAC (85:15) or
DOPE:DODAC:DMPE-PEG₂₀₀₀ (83:15:2). Fusion was assayed as described under
Figure 1 using 300 mM NaCl.

Figure 17 illustrates the recovery fusogenic activity after PEG removal.
Liposomes were prepared from either DOPE:DODAC:ceramide(C8:0)-PEG₂₀₀₀, 83:15:2
or DOPE:cholesterol:ceramide(C8:0)-PEG₂₀₀₀, 38:45:15:2. Fusion was assayed as
described under Figure 2 except that at the indicated times a 30 fold excess (over donors)
of liposomes composed of POPC or POPC:cholesterol (55:45) was added.

Figure 18 illustrates the effect of the lipid anchor on the rate of PEG-lipid
removal. Fluorescently labelled and unlabelled liposomes were prepared from
DOPE:DODAC:PEG-lipid, 83:15:2, using DMPE-PEG₂₀₀₀ (●), ceramide(egg)-PEG₂₀₀₀
or (C14:0) ceramide-PEG₂₀₀₀ (◆). Labelled liposomes were mixed with a 3 fold excess
of unlabelled liposomes and 300 mM NaCl in a cuvette in a dark water bath at 37°C. At
zero time a 13-fold excess (over labelled vesicles) of POPC liposomes was added and

the fluorescence intensity was measured at the indicated times. At the end of the assay Triton X-100 (0.5% final) was added to eliminate energy transfer and the % fusion was calculated from the change in efficiency of energy transfer. Maximum fusion was calculated from a standard curve of energy transfer efficiency against the molar fraction of Rh-PE in the membrane assuming complete mixing of labelled and unlabelled liposomes.

Figure 19 illustrates the inhibition of fusion between DOPE:cholesterol:DODAC liposomes and anionic liposomes by PEG-ceramide. Liposomes were prepared from DOPE:cholesterol:DODAC, 40:45:15 (no PEG) or DOPE:cholesterol:DODAC:(C14:0) ceramide-PEG₂₀₀₀, 36:45:15:4 (4% PEG). Acceptor liposomes were prepared from DOPE:cholesterol:POPS, 25:45:30. A three-fold excess of acceptors was added to labelled vesicles after 30s and the fluorescence monitored at 517 nm with excitation at 465 nm.

Figure 20 illustrates the recovery of fusogenic activity upon PEG removal. Donor liposomes (50 μ M) were prepared from DOPE:cholesterol:DODAC:(C14:0)ceramide-PEG₂₀₀₀, 36:45:15:4 and mixed with acceptor liposomes (150 μ M) prepared from DOPE:cholesterol:POPS, 25:45:30. At zero time either 1mM POPC:cholesterol liposomes (\blacktriangle) or an equivalent volume of buffer (\bullet) was added. Fluorescence was monitored at 517 nm with excitation at 465nm.

Figure 21 illustrates the inhibition of fusion between DOPE:cholesterol:DODAC liposomes and erythrocyte ghosts by, PEG-ceramide. Liposomes were prepared from DOPE:cholesterol:DODAC, 40:45:15 (no PEG) or DOPE:cholesterol:DODAC:(C14:0)ceramide-PEG₂₀₀₀, 36:45:15:4 (4% PEG). Ghosts (50 μ M phospholipid) were added to donors (50 μ M total lipid) after 30s and the fluorescence monitored at 517 nm with excitation at 465 nm.

Figure 22 illustrates the fusion of fluorescent liposomes composed of DOPE:cholesterol:DODAC (40:45:15) or DOPE:cholesterol:DODAC:PEG-ceramide (35:45:15:5). LUVs composed of DOPE:cholesterol:DODAC (40:45:15) fused with RBCs (panels a and b); incorporation of PEG-ceramide (C8:0) into the LUVs at 5 mol% blocked fusion (panels c and d); however, when an exogenous sink for the PEG-ceramide was included, fusogenic activity was recovered within minutes (panels e and f). Panels a, c and e are views under phase contrast, and panels b,d and f are the same fields view under fluorescent light.

Figure 23 illustrates the results when PEG-ceramides with longer fatty amide chains (C14:0) are used and the liposomes are pre-incubation with an exogenous sink prior to the addition of the RBCs. No fusion was observed after pre-incubation of the fusogenic LUVs with the sink for five minutes prior to addition of RBC (panels a and b); after a 1 hour pre-incubation, some fusion with RBCs was observed (panels c and d); however, with longer incubations times (2 hours), the pattern of fluorescent labeling changed and extensive punctate fluorescence was observed (panels e and f). Panels a, c and e are views under phase contrast, and panels b, d and f are the same fields view under fluorescent light.

Figure 24 illustrates the results when PEG-ceramides with longer fatty amide chains (C20:0) are used and the liposomes are preincubation with an exogenous sink prior to the addition of the RBCs. No fusion was observed after pre-incubation of the LUVs with the sink for five minutes (panels a and b), 1 hour (panels c and d) or 2 hours (panels e and f). Panels a, c and e are views under phase contrast, and panels b, d and f are the same fields view under fluorescent light.

Figure 25 graphically illustrates the fusion of PEG₂₀₀₀-DMPE and PEG₂₀₀₀-Ceramide (C14:0) containing vesicles with an anionic target.

Figure 26 graphically shows the effect of increasing concentrations of PEG-Ceramide (C20) on liposome clearance from the blood. ³H-labeled liposomes composed of DOPE (dioleoylphosphatidylethanolamine), 15 mol% DODAC (N,N-dioleoyl-N,N-dimethylammonium chloride) and the indicated concentrations of PEG-Ceramide (C20) were injected i.v. into mice. Biodistribution was examined at 1 hour after injection, and the data were expressed as a percentage of the injected dose in the blood (upper panel) and liver (lower panel) with SD (standard deviation) (n=3).

Figure 27 graphically illustrates the effect of increasing concentrations of DODAC on the biodistribution of liposomes in the blood. ³H-labeled liposomes composed of DOPE, 10 (open squares) or 30 (open triangles) mol% PEG-Ceramide (C20), and the indicated concentration of DODAC were injected i.v. into mice. Biodistribution was examined at 1 hour after injection, and the data were expressed as a percentage of the injected dose in the blood.

Figure 28 graphically shows the liposome levels in the blood and liver at different times after injection. ³H-labeled liposomes composed of DOPE/DODAC (85:15 mol/mol) (open circles with 0% PEG-Ceramide (C20)), DOPE/DODAC/PEG-Ceramide

(C20) (75:15:10 mol/mol/mol) (open squares with 10% PEG-Ceramide (C20)), and DOPE/DODAC/PEG-Ceramide (C20) (55:15:30 mol/mol/mol) (open triangles with 30% PEG-Ceramide (C20)) were injected i.v. into mice. Biodistribution was examined at indicated times, and the data were expressed as a percentage of the injected dose in the blood (upper panel) and in the liver (lower panel) with SD (n=3).

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

In one embodiment of the present invention, a fusogenic liposome is provided, the fusogenic liposome comprising: a lipid capable of adopting a non-lamellar phase, yet capable of assuming a bilayer structure in the presence of a bilayer stabilizing component; and a bilayer stabilizing component reversibly associated with the lipid to stabilize the lipid in a bilayer structure. Such fusogenic liposomes are advantageous because the rate at which they become fusogenic can be not only predetermined, but varied as required over a time scale of a few minutes to several tens of hours. It has been found, for example, that by controlling the composition and concentration of the bilayer stabilizing component, one can control the rate at which the bilayer stabilizing component exchanges out of the liposome and, in turn, the rate at which the liposome becomes fusogenic.

The polymorphic behavior of lipids in organized assemblies can be explained qualitatively in terms of the dynamic molecular shape concept (*see*, Cullis, *et al.*, in "Membrane Fusion" (Wilschut, J. and D. Hoekstra (eds.), Marcel Dekker, Inc., New York, (1991)). When the effective cross-sectional areas of the polar head group and the hydrophobic region buried within the membrane are similar then the lipids have a cylindrical shape and tend to adopt a bilayer conformation. Cone-shaped lipids which have polar head groups that are small relative to the hydrophobic component, such as unsaturated phosphatidylethanolamines, prefer non-bilayer phases such as inverted micelles or inverse hexagonal phase (H_{II}). Lipids with head groups that are large relative to their hydrophobic domain, such as lysophospholipids, have an inverted cone shape and tend to form micelles in aqueous solution. The phase preference of a mixed lipid system depends, therefore, on the contributions of all the components to the net dynamic molecular shape. As such, a combination of cone-shaped and inverted cone-shaped lipids

can adopt a bilayer conformation under conditions where either lipid in isolation cannot (see, Madden and Cullis, *Biochim. Biophys. Acta*, 684:149-153 (1982)).

A more formalized model is based on the intrinsic curvature hypothesis (see, e.g., Kirk, *et al.*, *Biochemistry*, 23:1093-1102 (1984)). This model explains phospholipid polymorphism in terms of two opposing forces. The natural tendency of a lipid monolayer to curl and adopt its intrinsic or equilibrium radius of curvature (R_0) which results in an elastically relaxed monolayer is opposed by the hydrocarbon packing constraints that result. Factors that decrease the intrinsic radius of curvature, such as increased volume occupied by the hydrocarbon chains when double bonds are introduced, tend to promote H_{II} phase formation. Conversely, an increase in the size of the headgroup increases R_0 and promotes bilayer formation or stabilization. Introduction of apolar lipids that can fill the voids between inverted lipid cylinders also promotes H_{II} phase formation (see, Gruner, *et al.*, *Proc. Natl. Acad. Sci. USA*, 82:3665-3669 (1989); Sjoland, *et al.*, *Biochemistry*, 28:1323-1329 (1989)).

Lipids which can be used to form the fusogenic liposomes of the present invention are those which adopt a non-lamellar phase under physiological conditions or under specific physiological conditions, e.g., in the presence of calcium ions, but which are capable of assuming a bilayer structure in the presence of a bilayer stabilizing component. Such lipids include, but are not limited to, phosphatidylethanolamines, ceramides, glycolipids, or mixtures thereof. Other lipids known to those of skill in the art to adopt a non-lamellar phase under physiological conditions can also be used. Moreover, it will be readily apparent to those of skill in the art that other lipids can be induced to adopt a non-lamellar phase by various non-physiological changes including, for example, changes in pH or ion concentration (e.g., in the presence of calcium ions) and, thus, they can also be used to form the fusogenic liposomes of the present invention. In a presently preferred embodiment, the fusogenic liposome is prepared from a phosphatidylethanolamine. The phosphatidylethanolamine can be saturated or unsaturated. In a presently preferred embodiment, the phosphatidylethanolamine is unsaturated. In an equally preferred embodiment, the fusogenic liposome is prepared from a mixture of a phosphatidylethanolamine (saturated or unsaturated) and a phosphatidylserine. In another equally preferred embodiment, the fusogenic liposome is prepared from a mixture of a phosphatidylethanolamine (saturated or unsaturated) and a cationic lipid.

Examples of suitable cationic lipids include, but are not limited to, the following: DC-Chol, 3 β -(N-(N',N'-dimethylaminoethane)carbonyl)cholesterol (*see*, Gao, *et al.*, *Biochem. Biophys. Res. Comm* 179:280-285 (1991)); DDAB, N,N-distearyl-N,N-dimethylammonium bromide; DMRIE, N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide; DODAC, N,N-dioleoyl-N,N-dimethylammonium chloride (*see*, commonly owned United States Patent Application Serial Number 08/316,399, filed September 30, 1994, which is incorporated herein by reference); DOGS, diheptadecylamidoglycyl spermidine; DOSPA, N-(1-(2,3-dioleoyloxy)propyl)-N-(2-(sperminecarboxamido)ethyl)-N,N-dimethylammonium trifluoroacetate; DOTAP, N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride; and DOTMA, N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride. In a presently preferred embodiment, N,N-dioleoyl-N,N-dimethylammonium chloride is used in combination with a phosphatidylethanolamine.

In accordance with the present invention, lipids adopting a non-lamellar phase under physiological conditions can be stabilized in a bilayer structure by bilayer stabilizing components which are either bilayer forming themselves, or which are of a complementary dynamic shape. The non-bilayer forming lipid is stabilized in the bilayer structure only when it is associated with, *i.e.*, in the presence of, the bilayer stabilizing component. In selecting an appropriate bilayer stabilizing component, it is imperative that the bilayer stabilizing component be capable of transferring out of the liposome, or of being chemically modified by endogenous systems such that, with time, it loses its ability to stabilize the lipid in a bilayer structure. Only when liposomal stability is lost or decreased can fusion of the liposome with the plasma membrane of the target cell occur. The bilayer stabilizing component is, therefore, "reversibly associated" with the lipid and only when it is associated with the lipid is the lipid constrained to adopt the bilayer structure under conditions where it would otherwise adopt a non-lamellar phase. As such, the bilayer stabilizing components of the present invention must be capable of stabilizing the lipid in a bilayer structure, yet they must be capable of exchanging out of the liposome, or of being chemically modified by endogenous systems so that, with time, they lose their ability to stabilize the lipid in a bilayer structure, thereby allowing the liposome to become fusogenic.

Examples of suitable bilayer stabilizing components include, but are not limited to, lipid, lipid-derivatives, detergents, proteins and peptides. In a presently

preferred embodiment, the bilayer stabilizing component is polyethyleneglycol conjugated to, *i.e.*, coupled to, a phosphatidylethanolamine. In an equally preferred embodiment, the bilayer stabilizing component is polyethyleneglycol conjugated to a ceramide.

Polyethyleneglycol can be conjugated to a phosphatidylethanolamine or, alternatively, to a ceramide using standard coupling reactions known to and used by those of skill in the art. In addition, preformed polyethyleneglycol-phosphatidylethanolamine conjugates are commercially available from Avanti Polar Lipids (Alabaster, Alabama).

Polyethyleneglycols of varying molecular weights can be used to form the bilayer stabilizing components of the present invention. Polyethyleneglycols of varying molecular weights are commercially available from a number of different sources or, alternatively, they can be synthesized using standard polymerization techniques well-known to those of skill in the art. In a presently preferred embodiment, the polyethylene glycol has a molecular weight ranging from about 200 to about 10,000, more preferably from about 1,000 to about 8,000, and even more preferably from about 2,000 to about 6,000. Generally, it has been found that increasing the molecular weight of the polyethyleneglycol reduces the concentration of the bilayer stabilizing component required to achieve stabilization.

Phosphatidylethanolamines having a variety of acyl chain groups of varying chain lengths and degrees of saturation can be conjugated to polyethyleneglycol to form the bilayer stabilizing component. Such phosphatidylethanolamines are commercially available, or can be isolated or synthesized using conventional techniques known to those of skill in the art. Phosphatidylethanolamines containing saturated or unsaturated fatty acids with carbon chain lengths in the range of C_{10} to C_{20} are preferred. Phosphatidylethanolamines with mono- or diunsaturated fatty acids and mixtures of saturated and unsaturated fatty acids can also be used. Suitable phosphatidylethanolamines include, but are not limited to, the following: dimyristoylphosphatidylethanolamine (DMPE), dipalmitoylphosphatidylethanolamine (DPPE), dioleoylphosphatidylethanolamine (DOPE) and distearoylphosphatidylethanolamine (DSPE).

As with the phosphatidylethanolamines, ceramides having a variety of acyl chain groups of varying chain lengths and degrees of saturation can be coupled to polyethyleneglycol to form the bilayer stabilizing component. It will be apparent to those of skill in the art that in contrast to the phosphatidylethanolamines, ceramides have only

one acyl group which can be readily varied in terms of its chain length and degree of saturation. Ceramides suitable for use in accordance with the present invention are commercially available. In addition, ceramides can be isolated, for example, from egg or brain using well-known isolation techniques or, alternatively, they can be synthesized using the methods and techniques disclosed in U.S. Patent Application Serial Number 08/316,429, filed September 30, 1994, and U.S. Patent Application filed on an even date herewith and bearing Attorney Docket No. 16303-001010, the teachings of which are incorporated herein by reference. Using the synthetic routes set forth in the foregoing application, ceramides having saturated or unsaturated fatty acids with carbon chain lengths in the range of C₂ to C₃₁ can be prepared.

In addition to the foregoing, detergents, proteins and peptides can be used as bilayer stabilizing components. Detergents which can be used as bilayer stabilizing components include, but are not limited to, Triton X-100, deoxycholate, octylglucoside and lyso-phosphatidylcholine. Proteins which can be used as bilayer stabilizing components include, but are not limited to, glycophorin and cytochrome oxidase. Cleavage of the protein, by endogenous proteases, resulting in the loss of the bulky domain external to the bilayer would be expected to reduce the bilayer stabilizing ability of the protein. In addition, peptides which can be used as bilayer stabilizing components include, for example, the pentadecapeptide, alanine-(aminobutyric acid-alanine)₁₄. This peptide can be coupled, for example, to polyethyleneglycol which would promote its transfer out of the bilayer. Alternatively, peptides such as cardiotoxin and melittin, both of which are known to induce non-lamellar phases in bilayers, can be coupled to PEG and might thereby be converted to bilayer stabilizers in much the same way that PE is converted from a non-lamellar phase preferring lipid to a bilayer stabilizer when it is coupled to PEG. If the bond between the peptide and the PEG is labile, then cleavage of the bond would result in the loss of the bilayer stabilizing ability and in the restoration of a non-lamellar phase, thereby causing the liposome to become fusogenic.

Typically, the bilayer stabilizing component is present at a concentration ranging from about 0.05 mole percent to about 50 mole percent. In a presently preferred embodiment, the bilayer stabilizing component is present at a concentration ranging from 0.05 mole percent to about 25 mole percent. In an even more preferred embodiment, the bilayer stabilizing component is present at a concentration ranging from 0.05 mole percent to about 15 mole percent. One of ordinary skill in the art will appreciate that the

concentration of the bilayer stabilizing component can be varied depending on the bilayer stabilizing component employed and the rate at which the liposome is to become fusogenic.

By controlling the composition and concentration of the bilayer stabilizing component, one can control the rate at which the bilayer stabilizing component exchanges out of the liposome and, in turn, the rate at which the liposome becomes fusogenic. For instance, when a polyethyleneglycol-phosphatidylethanolamine conjugate or a polyethyleneglycol-ceramide conjugate is used as the bilayer stabilizing component, the rate at which the liposome becomes fusogenic can be varied, for example, by varying the concentration of the bilayer stabilizing component, by varying the molecular weight of the polyethyleneglycol, or by varying the chain length and degree of saturation of the acyl chain groups on the phosphatidylethanolamine or the ceramide. In addition, other variables including, for example, pH, temperature, ionic strength, *etc.* can be used to vary and/or control the rate at which the liposome becomes fusogenic. Other methods which can be used to control the rate at which the liposome becomes fusogenic will become apparent to those of skill in the art upon reading this disclosure.

In a presently preferred embodiment, the fusogenic liposomes contain cholesterol. It has been determined that when cholesterol-free liposomes are used *in vivo*, they have a tendency to absorb cholesterol from plasma lipoproteins and cell membranes. Since this absorption of cholesterol could, in theory, change the fusogenic behavior of the liposomes, cholesterol can be included in the fusogenic liposomes of the present invention so that little or no net transfer of cholesterol occurs *in vivo*.

Cholesterol, if included, is generally present at a concentration ranging from 0.02 mole percent to about 50 mole percent and, more preferably, at a concentration ranging from about 35 mole percent to about 45 mole percent.

A variety of methods are available for preparing liposomes as described in, *e.g.*, Szoka *et al.*, *Ann. Rev. Biophys. Bioeng.* 9:467 (1980), U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, 4,946,787, PCT Publication No. WO 91/17424, Deamer and Bangham, *Biochim. Biophys. Acta*, 443:629-634 (1976); Fraley, *et al.*, *Proc. Natl. Acad. Sci. USA* 76:3348-3352 (1979); Hope, *et al.*, *Biochim. Biophys. Acta* 812:55-65 (1985); Mayer, *et al.*, *Biochim. Biophys. Acta* 858:161-168 (1986); Williams, *et al.*, *Proc. Natl. Acad. Sci.*

USA 85:242-246 (1988); the text *Liposomes*, (Marc J. Ostro (ed.), Marcel Dekker, Inc., New York, 1983, Chapter 1); and Hope, *et al.*, *Chem. Phys. Lip.* 40:89 (1986), all of which are incorporated herein by reference. Suitable methods include, for example, sonication, extrusion, high pressure/homogenization, microfluidization, detergent dialysis, calcium-induced fusion of small liposome vesicles and ether-fusion methods, all of which are well known in the art. One method produces multilamellar vesicles of heterogeneous sizes. In this method, the vesicle-forming lipids are dissolved in a suitable organic solvent or solvent system and dried under vacuum or an inert gas to form a thin lipid film. If desired, the film may be redissolved in a suitable solvent, such as tertiary butanol, and then lyophilized to form a more homogeneous lipid mixture which is in a more easily hydrated powder-like form. This film is covered with an aqueous buffered solution and allowed to hydrate, typically over a 15-60 minute period with agitation. The size distribution of the resulting multilamellar vesicles can be shifted toward smaller sizes by hydrating the lipids under more vigorous agitation conditions or by adding solubilizing detergents such as deoxycholate.

Unilamellar vesicles are generally prepared by sonication or extrusion. Sonication is generally preformed with a tip sonifier, such as a Branson tip sonifier, in an ice bath. Typically, the suspension is subjected to several sonication cycles. Extrusion can be carried out by biomembrane extruders, such as the Lipex Biomembrane Extruder. Defined pore size in the extrusion filters can generate unilamellar liposomal vesicles of specific sizes. The liposomes can also be formed by extrusion through an asymmetric ceramic filter, such as a Ceraflow Microfilter, commercially available from the Norton Company, Worcester MA.

Following liposome preparation, the liposomes may be sized to achieve a desired size range and relatively narrow distribution of liposome sizes. A size range of about 0.05 microns to about 0.20 microns allows the liposome suspension to be sterilized by filtration through a conventional filter, typically a 0.22 micron filter. The filter sterilization method can be carried out on a high through-put basis if the liposomes have been sized down to about 0.05 microns to about 0.20 microns.

Several techniques are available for sizing liposomes to a desired size. One sizing method is described in U.S. Pat. No. 4,737,323, incorporated herein by reference. Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles less than about 0.05

microns in size. Homogenization is another method which relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0.1 and 0.5 microns, are observed. In both of these methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination. In addition, the size of the liposomal vesicle can be determined by quasi-electric light scattering (QELS) as described in Bloomfield, *Ann. Rev. Biophys. Bioeng.* 10:421-450 (1981), incorporated herein by reference. Average liposome diameter can be reduced by sonication of formed liposomes. Intermittent sonication cycles can be alternated with QELS assessment to guide efficient liposome synthesis.

Extrusion of liposome through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing liposome sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size. For use in the present inventions, liposomes having a size of from about 0.05 microns to about 0.45 microns are preferred.

For the delivery of therapeutic agents, the fusogenic liposomes of the present invention can be loaded with a therapeutic agent and administered to the subject requiring treatment. The therapeutic agents which can be administered using the fusogenic liposomes of the present invention can be any of a variety of drugs, peptides, proteins, DNA, RNA or other bioactive molecules. Moreover, cationic lipids may be used in the delivery of therapeutic genes or oligonucleotides intended to induce or to block production of some protein within the cell. Nucleic acid is negatively charged and must be combined with a positively charged entity to form a complex suitable for formulation and cellular delivery.

Cationic lipids have been used in the transfection of cells *in vitro* and *in vivo* (Wang, C-Y, Huang L., "pH sensitive immunoliposomes mediate target cell-specific delivery and controlled expression of a foreign gene in mouse," *Proc. Natl. Acad. Sci. USA*, 1987; 84:7851-7855 and Hyde, S.C., Gill, D.R., Higgins, C.F., *et al.*, "Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy," *Nature*, 1993; 362:250-255). The efficiency of this transfection has often been

less than desired, for various reasons. One is the tendency for cationic lipids complexed to nucleic acid to form unsatisfactory carriers. These carriers are improved by the addition of PEG-modified lipids and, in particular, PEG-modified ceramide lipids. The addition of PEG-modified lipids prevents particle aggregation and provides a means for increasing circulation lifetime and increasing the delivery of the lipid-nucleic acid particles to the target cells. Moreover, it has been found that cationic lipids fuse more readily with the target cells and, thus, the addition of neutrally charged PEG-modified ceramide lipids does not mask or diminish the positive charge of the carrier liposomes.

Cationic lipids useful in producing lipid based carriers for gene and oligonucleotide delivery include, but are not limited to, 3β -(N-(N',N'-dimethylaminoethane)carbonyl)cholesterol (DC-Chol); N,N-distearyl-N,N-dimethylammonium bromide (DDAB); N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE); diheptadecylamidoglycyl spermidine (DOGS); N-(1-(2,3-dioleoyloxy)propyl)-N-(2-(sperminecarboxamido)ethyl)-N,N-dimethylammonium trifluoroacetate (DOSPA); N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP); N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA); N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC); LIPOFECTIN, a commercially available cationic lipid comprising DOTMA and DOPE (GIBCO/BRL, Grand Island, N.Y.) (U.S. Patent Nos. 4,897,355; 4,946,787; and 5,208,036 issued to Epstein, *et al.*); LIPOFECTACE or DDAB (dimethyldioctadecyl ammonium bromide) (U.S. Patent No. 5,279,883 issued to Rose); LIPOFECTAMINE, a commercially available cationic lipid composed of DOSPA and DOPE (GIBCO/BRL, Grand Island, N.Y.); TRANSFECTAM, a commercially available cationic lipid comprising DOGS (Promega Corp., Madison, WI).

Any variety of drugs which are selected to be an appropriate treatment for the disease to be treated in the tissue can be administered using the fusogenic liposomes of the present invention. Often the drug will be an antineoplastic agent, such as vincristine, doxorubicin, cisplatin, bleomycin, cyclophosphamide, methotrexate, streptozotocin, and the like. It may also be desirable to deliver anti-infective agents to specific tissues by the present methods. The compositions of the present invention can also be used for the selective delivery of other drugs including, but not limited to local anesthetics, *e.g.*, dibucaine and chlorpromazine; beta-adrenergic blockers, *e.g.*, propranolol, timolol and labetalol; antihypertensive agents, *e.g.*, clonidine and

hydalazine; anti-depressants, *e.g.*, imipramine, amitriptyline and doxepim; anti-convulsants, *e.g.*, phenytoin; antihistamines, *e.g.*, diphenhydramine, chlorpheniramine and promethazine; antibacterial agents, *e.g.*, gentamycin; antifungal agents, *e.g.*, miconazole, terconazole, econazole, isoconazole, butaconazole, clotrimazole, itraconazole, nystatin, naftifine and amphotericin B; antiparasitic agents, hormones, hormone antagonists, immunomodulators, neurotransmitter antagonists, antiglaucoma agents, vitamins, narcotics, and imaging agents. Other particular drugs which can be selectively administered by the compositions of the present invention will be well known to those of skill in the art. Additionally, two or more therapeutic agents may be administered simultaneously if desired, where such agents produce complementary or synergistic effects.

Methods of loading conventional drugs into liposomes include an encapsulation technique and the transmembrane potential loading method. In one encapsulation technique, the drug and liposome components are dissolved in an organic solvent in which all species are miscible and concentrated to a dry film. A buffer is then added to the dried film and liposomes are formed having the drug incorporated into the vesicle walls. Alternatively, the drug can be placed into a buffer and added to a dried film of only lipid components. In this manner, the drug will become encapsulated in the aqueous interior of the liposome. The buffer which is used in the formation of the liposomes can be any biologically compatible buffer solution of, for example, isotonic saline, phosphate buffered saline, or other low ionic strength buffers. Generally, the drug will be present in an amount of from about 0.01 ng/mL to about 50 mg/mL. The resulting liposomes with the drug incorporated in the aqueous interior or in the membrane are then optionally sized as described above.

Transmembrane potential loading has been described in detail in U.S. Patent No. 4,885,172, U.S. Patent No. 5,059,421, and U.S. Patent No. 5,171,578, the contents of which are incorporated herein by reference. Briefly, the transmembrane potential loading method can be used with essentially any conventional drug which exhibits weak acid or weak base characteristics. Preferably, the drug will be lipophilic so that it will partition into the liposome membrane. A pH gradient is created across the bilayers of the liposomes or protein-liposome complexes, and the drug is loaded into the liposome in response to the pH gradient. The pH gradient is generated by creating a proton gradient across the membrane either by making the interior more

acidic or basic than the exterior (Harrigan, *et al.*, *Biochem. Biophys. Acta.* 1149:329-339 (1993), the teachings of which are incorporated herein by reference), or by establishing an ion gradient employing ionizable agents, such as ammonium salts, which leads to the generation of a pH gradient (See, U.S. Patent No. 5,316,771 (Barenholz), the teachings of which are incorporated herein by reference).

In certain embodiments of the present invention, it is desirable to target the liposomes of the invention using targeting moieties that are specific to a particular cell type, tissue, and the like. Targeting of liposomes using a variety of targeting moieties (*e.g.*, ligands, receptors and monoclonal antibodies) has been previously described (*see, e.g.*, U.S. Patent Nos. 4,957,773 and 4,603,044, both of which are incorporated herein by reference).

Examples of targeting moieties include monoclonal antibodies specific to antigens associated with neoplasms, such as prostate cancer specific antigen. Tumors can also be diagnosed by detecting gene products resulting from the activation or overexpression of oncogenes, such as *ras* or *c-erbB2*. In addition, many tumors express antigens normally expressed by fetal tissue, such as the alphafetoprotein (AFP) and carcinoembryonic antigen (CEA). Sites of viral infection can be diagnosed using various viral antigens such as hepatitis B core and surface antigens (HBVc, HBVs) hepatitis C antigens, Epstein-Barr virus antigens, human immunodeficiency type-1 virus (HIV1) and papilloma virus antigens. Inflammation can be detected using molecules specifically recognized by surface molecules which are expressed at sites of inflammation such as integrins (*e.g.*, VCAM-1), selectin receptors (*e.g.*, ELAM-1) and the like.

Standard methods for coupling targeting agents to liposomes can be used. These methods generally involve incorporation into liposomes lipid components, *e.g.*, phosphatidylethanolamine, which can be activated for attachment of targeting agents, or derivatized lipophilic compounds, such as lipid derivatized bleomycin. Antibody targeted liposomes can be constructed using, for instance, liposomes which incorporate protein A (*see, Renneisen, et al.*, *J. Biol. Chem.*, 265:16337-16342 (1990) and Leonetti, *et al.*, *Proc. Natl. Acad. Sci. (USA)* 87:2448-2451 (1990), both of which are incorporated herein by reference).

Targeting mechanisms generally require that the targeting agents be positioned on the surface of the liposome in such a manner that the target moieties are available for interaction with the target, for example, a cell surface receptor. The

liposome is typically fashioned in such a way that a connector portion is first incorporated into the membrane at the time of forming the membrane. The connector portion must have a lipophilic portion which is firmly embedded and anchored in the membrane. It must also have a hydrophilic portion which is chemically available on the aqueous surface of the liposome. The hydrophilic portion is selected so that it will be chemically suitable to form a stable chemical bond with the targeting agent which is added later. Therefore, the connector molecule must have both a lipophilic anchor and a hydrophilic reactive group suitable for reacting with the target agent and holding the target agent in its correct position, extended out from the liposome's surface. In some cases it is possible to attach the target agent to the connector molecule directly, but in most instances it is more suitable to use a third molecule to act as a chemical bridge, thus linking the connector molecule which is in the membrane with the target agent which is extended, three dimensionally, off of the vesicle surface.

Following a separation step as may be necessary to remove free drug from the medium containing the liposome, the liposome suspension is brought to a desired concentration in a pharmaceutically acceptable carrier for administration to the patient or host cells. Many pharmaceutically acceptable carriers may be employed in the compositions and methods of the present invention. Suitable formulations for use in the present invention are found in *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). A variety of aqueous carriers may be used, for example, water, buffered water, 0.4% saline, 0.3% glycine, and the like, and may include glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, etc. Generally, normal buffered saline (135-150 mM NaCl) will be employed as the pharmaceutically acceptable carrier, but other suitable carriers will suffice. These compositions can be sterilized by conventional liposomal sterilization techniques, such as filtration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc. These compositions can be sterilized using the techniques referred to above or, alternatively, they can be produced under sterile conditions. The resulting aqueous solutions may be packaged for use or filtered

under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration.

5 The concentration of liposomes in the carrier may vary. Generally, the concentration will be about 20-200 mg/ml, usually about 50-150 mg/ml, and most usually about 75-125 mg/ml, *e.g.*, about 100 mg/ml. Persons of skill may vary these concentrations to optimize treatment with different liposome components or for particular patients. For example, the concentration may be increased to lower the fluid load associated with treatment.

10 The present invention also provides methods for introducing therapeutic compounds into cells of a host. The methods generally comprise administering to the host a fusogenic liposome containing the therapeutic compound, wherein the fusogenic liposome comprises a bilayer stabilizing component and a lipid which adopts a non-lamellar phase under physiological conditions, yet which is capable of assuming a bilayer structure in the presence of said bilayer stabilizing component. The host may be a
15 variety of animals, including humans, non-human primates, avian species, equine species, bovine species, swine, lagomorpha, rodents, and the like.

The cells of the host are usually exposed to the liposomal preparations of the invention by *in vivo* administration of the formulations, but *ex vivo* exposure of the cells to the liposomes is also feasible. *In vivo* exposure is obtained by administration of
20 the liposomes to host. The liposomes may be administered in many ways. These include parenteral routes of administration, such as intravenous, intramuscular, subcutaneous, and intraarterial. Generally, the liposomes will be administered intravenously or in some cases via inhalation. Often, the liposomes will be administered into a large central vein, such as the superior vena cava or inferior vena cava, to allow
25 highly concentrated solutions to be administered into large volume and flow vessels. The liposomes may be administered intraarterially following vascular procedures to deliver a high concentration directly to an affected vessel. In some instances, the liposomes may be administered orally or transdermally, although the advantages of the present invention are best realized by parenteral administration. The liposomes may also be incorporated
30 into implantable devices for long duration release following placement.

As described above, the liposomes will generally be administered intravenously or via inhalation in the methods of the present invention. Often multiple treatments will be given to the patient. The dosage schedule of the treatments will be

determined by the disease and the patient's condition. Standard treatments with therapeutic compounds that are well known in the art may serve as a guide to treatment with liposomes containing the therapeutic compounds. The duration and schedule of treatments may be varied by methods well known to those of skill, but the increased circulation time and decreased in liposome leakage will generally allow the dosages to be adjusted downward from those previously employed. The dose of liposomes of the present invention may vary depending on the clinical condition and size of the animal or patient receiving treatment. The standard dose of the therapeutic compound when not encapsulated may serve as a guide to the dose of the liposome-encapsulated compound. The dose will typically be constant over the course of treatment, although in some cases the dose may vary. Standard physiological parameters may be assessed during treatment that may be used to alter the dose of the liposomes of the invention.

Liposome charge is an important determinant in liposome clearance from the blood, with negatively charged liposomes being taken up more rapidly by the reticuloendothelial system (Juliano, *Biochem. Biophys. Res. Commun.* 63:651 (1975)) and, thus, having shorter half-lives in the bloodstream. Liposomes with prolonged circulation half-lives are typically desirable for therapeutic and diagnostic uses. To maximize circulation half-lives, the bilayer stabilizing component should be a hydrophilic polymer, e.g., PEG, conjugated to lipid anchors, e.g., PEs, having long, saturated hydrocarbon chains (C18 > C16 > C14) as these conjugates provide a longer lasting steric barrier. As such, by varying the charge in addition to the foregoing factors, one of skill in the art can regulate the rate at which the liposomes of the present invention become fusogenic.

Additionally, the liposome suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as alphanatocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

The invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner.

EXAMPLES**I. MATERIALS AND GENERAL METHODS****5 A. Materials**

All phospholipids including fluorescent probes and PEG-PE conjugates were purchased from Avanti Polar Lipids, Birmingham, Alabama, USA. 1-O-methyl-(poly(ethoxy)-O-succinyl-O-(egg)ceramide which was a gift from Dr L. Choi of Inex Pharmaceuticals Corp., Vancouver, BC, Canada. Di-[1-¹⁴C]-palmitoylphosphatidyl-
10 choline was purchased from Du Pont, Mississauga, Ont., Canada. [³H]-DSPE-PEG₂₀₀₀ was synthesized as described previously (Parr, *et al.*, *Biochim. Biophys. Acta*, 1195: 21-30 (1994)). Other reagents were purchased from Sigma Chemical Co., St Louis, Missouri, USA.

15 B. Preparation of multilamellar vesicles and large unilamellar vesicles

Lipid components were mixed in 1-2 ml of benzene:methanol (95:5, v/v) and then lyophilized for a minimum of 5 hours at a pressure of <60 millitorr using a Virtis lyophilizer equipped with a liquid N₂ trap. Multilamellar vesicles (MLVs) were prepared by hydrating the dry lipid mixtures in 150 mM NaCl, buffered with 10 mM
20 Hepes-NaOH, pH 7.4 (Hepes-buffered saline, HBS). Mixtures were vortexed to assist hydration. To produce large unilamellar vesicles (LUVs), MLVs were first frozen in liquid nitrogen and then thawed at 30°C five times. LUVs were produced by extrusion of the frozen and thawed MLVs ten times through 2 stacked polycarbonate filters of 100 nm pore size at 30°C and pressures of 200-500 psi (Hope, *et al.*, *Biochim. Biophys.*
25 *Acta*, 812:55-65 (1985)).

C. ³¹P-NMR spectroscopy

³¹P-NMR spectra were obtained using a temperature controlled Bruker MSL200 spectrometer operating at 81MHz. Free induction decays were accumulated for
30 2000 transients using a 4 μs, 90° pulse, 1 sec. interpulse delay, 20 KHz sweep width and Waltz decoupling. A 50 Hz line broadening was applied to the data prior to Fourier transformation. Samples were allowed to equilibrate at the indicated temperature for 30 minutes prior to data accumulation. Lipid concentrations of 30-70 mM were used.

D. *Freeze-fracture Electron Microscopy*

MLVs were prepared by hydrating a mixture of DOPE:cholesterol:DOPE-PEG₂₀₀₀ (1:1:0:1) with HBS. A portion of the mixture was extruded as described above to produce LUVs. Glycerol was added to both MLVs and LUVs to a final concentration of 25% and samples were rapidly frozen in liquid freon. The samples were fractured at -110°C and < 10⁻⁶ torr in a Balzers BAF400 unit. Replicas were prepared by shadowing at 45° with a 2 nm layer of platinum and coating at 90° with a 20 nm layer of carbon. The replicas were cleaned by soaking in hypochlorite solution for up to 48 hrs and were visualized in a Jeol JEM-1200 EX electron microscope.

E. *Gel Filtration of LUVs and Micelles*

LUVs composed of DOPE:cholesterol:DSPE-PEG₂₀₀₀ (1:1:0:1) with trace amounts of ¹⁴C-DPPC and ³H-DSPE-PEG₂₀₀₀ were chromatographed at a flow rate of approximately 0.5 ml/min on a column of Sepharose CL-4B was pretreated with 10mg of eggPC, which had been suspended in HBS by bath sonication, to eliminate non-specific adsorption of lipid to the column. Micelles were prepared by hydrating DSPE-PEG₂₀₀₀ containing a trace amount of ³H-DSPE-PEG₂₀₀₀ with HBS and chromatographed as described for LUVs.

F. *Lipid Mixing Assays*

Lipid mixtures were prepared as described for NMR measurements. The resultant multilamellar vesicles (MLV) were frozen in liquid nitrogen and then thawed at 30°C five times. Large unilamellar vesicles (LUV) were produced by extrusion of the frozen and thawed MLV ten times through 2 stacked polycarbonate filters of 100 nm pore size at 30°C and pressures of 200-500 psi (Hope, *et al.*, *Biochim. Biophys. Acta* 812:55-65 (1985)).

Lipid mixing was measured by a modification of the fluorescence resonance energy transfer (FRET) assay of Struck, *et al.* (*Biochemistry* 20:4093-4099 (1981)). LUVs were prepared containing the fluorescent lipids, N-(7-nitro-2-1,3-benzoxadiazol-4-yl)-dioleoylphosphatidylethanolamine (NBD-PE) and N-(lissamine rhodamine B sulfonyl)-dipalmitoylphosphatidylethanolamine (Rh-PE) at 0.5 mol%. LUVs (50-60 μM) and a three-fold excess of unlabelled target vesicles were mixed in the fluorimeter at 37°C for short term assays (≤ 1 hour), or in sealed cuvettes in a dark

water bath at 37°C for longer assays. For measurements of fusion after PEG-lipid transfer, an excess of liposomes prepared from 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) was added as a sink for the PEG-lipid. Fluorescence emission intensity was measured at 517 nm with excitation at 465 nm both before and after the addition of Triton X-100 (final concentration of 0.5% or 1% when POPC sink was used). Data is presented as either uncorrected fluorescence intensity for short term assays (≤ 1 hour) or as percentage fusion. Light scattering controls were performed by replacing LUVs labelled with 0.5 mol% probes with unlabelled vesicles. Maximum fusion was determined using mock fused vesicles containing 0.125 mol% of each fluorescent probe.

The percentage fusion was calculated as:

$$\% \text{ Fusion} = \frac{\frac{(F_{(t)} - L_{(t)})}{(F_T - L_T)} - \frac{(F_0 - L_0)}{(F_T - L_T)}}{\frac{(M_{(t)} - L_{(t)})}{(M_T - L_T)} - \frac{(F_0 - L_0)}{(F_T - L_T)}} \times 100$$

where F_{∞} = fluorescence intensity at time t ; F_0 = fluorescence intensity at zero time; F_T = fluorescence intensity in the presence of Triton X-100. M and L represent the same measurements for the mock fused control and the light scattering control respectively. Changes in fluorescence of the mock fused control indicated that exchange of the fluorescent probes over 24 hours accounted for 10% of the fluorescence change observed, but was negligible over the first hour.

G. Fusion of Liposomes with Red Blood Cells

LUVs composed of DOPE:cholesterol:DODAC (40:45:15) or DOPE:cholesterol:DODAC:PEG-ceramide (35:45:15) were prepared by standard extrusion techniques. LUVs also contained 1 mol% rhodamine-PE. LUVs (200 μ M) were incubated at 37°C with 50 μ l packed RBCs in a final volume of 1 ml. For assays of fusion after PEG-lipid exchange, a sink of 2 mM POPC:cholesterol (55:45) was included. In some assays, the fusogenic liposomes were pre-incubated with the sink before being mixed with the RBCs (See, figure legends for Figures 22-24). Aliquots of the mixtures were transferred to glass microscope slides, covered with cover slips and examined by phase contrast and fluorescent microscopy. Fusion was assessed as fluorescent labeling of the RBC plasma membranes. For Figures 22-24, fluorescent

liposomes were incubated with POPC:cholesterol liposomes and/or RBCs as described in section "L," *infra*. Panels a,c and e of Figures 22-24 are views under phase contrast, whereas panels b,d and f of Figures 22-24 are the same fields viewed under fluorescent light.

H. Other Procedures

Phospholipid concentrations were determined by assaying for phosphate using the method of Fiske and Subbarow (*J. Biol. Chem.*, 66:375-400 (1925)).

Liposome size distributions were determined by quasi-elastic light scattering (QELS) using a Nicomp model 370 particle sizer.

II. EXPERIMENTAL FINDINGS

A. Influence of BSC on the polymorphic phase properties of an equimolar mixture of DOPE and cholesterol

³¹P-NMR was used to examine the effect of bilayer stabilizing component (BSC), in this instance poly-(ethyleneglycol)₂₀₀₀ conjugated to DOPE (*i.e.*, DOPE-PEG₂₀₀₀), on the phase preference of an equimolar mixture of DOPE and cholesterol (Figure 1). In the absence of BSC, the mixture adopted an inverse hexagonal phase (H_{II}) at 20°C as determined from the characteristic ³¹P-NMR lineshape with a low field peak and high field shoulder (Cullis and deKruijff, *Biochim. Biophys. Acta* 559:399-420 (1979)). As the amount of BSC in the mixture was increased, the peak corresponding to H_{II} phase phospholipid disappeared and a high field peak with a low field shoulder, characteristic of bilayer phase phospholipid (Cullis and deKruijff, *supra*, 1979) appeared. When DOPE-PEG₂₀₀₀ was present at 20 mol% of phospholipid, the mixture was almost exclusively bilayer with no evidence of H_{II} phase lipid.

In addition to the peaks corresponding to H_{II} phase and bilayer phase, a third peak indicative of isotropic motional averaging was observed in the presence of BSC (Figure 1). The size of the isotropic signal varied with the amount of BSC present and, as shown in subsequent Figures, the nature of the BSC species. The signal was largest at concentrations of BSC that allowed H_{II} and bilayer phases to co-exist and diminished when either H_{II} or bilayer phase predominated. Such a signal may be produced by a number of phospholipid phases which allow isotropic motional averaging

on the NMR timescale, including micellar, small vesicular, cubic and rhombic phase phospholipids.

5 **B. *The influence of BSC on the thermotropic properties of an equimolar mixture of DOPE and cholesterol***

Figure 2 illustrates the effect of temperature on the phase properties of mixtures of DOPE, cholesterol and BSC. When DOPE-PEG₂₀₀₀ was present at 9 mol%, there was a large isotropic signal which dominated the spectrum at all temperatures. The predominant, non-isotropic phase at 0°C was bilayer. However, as the temperature was increased the high field peak diminished and a shoulder corresponding to the low field peak of the H_n phase appeared. The apparent bilayer to hexagonal phase transition occurred at 40-50°C, but was almost obscured by the large isotropic signal. DOPE on its own exhibits a sharp transition over an interval of approximately 10°C (see, Figure 1 in Tilcock, *et al.*, *Biochemistry* 21:4596-4601 (1982)). The transition in mixtures of DOPE, cholesterol and BSC was slow in comparison with both phases present over a temperature range of almost 40°C (See also, Figure 3).

The mixture was stabilized in the bilayer conformation over the same temperature range when the BSC content was increased to 20 mol% (Figure 2). There was no evidence of phospholipid in the H_n phase. In addition, the isotropic signal was markedly reduced at the higher BSC concentration at all temperatures studied. The amount of lipid experiencing isotropic motional averaging increased as the temperature increased for both concentrations of BSC.

25 **C. *The effect of head group size on the bilayer stabilizing properties of BSCs***

The influence of head group size on the bilayer stabilizing properties of BSCs is illustrated in Figure 3. DOPE-PEG₂₀₀₀ at 5 mol% had limited bilayer stabilizing ability. A broad bilayer to H_n transition was centered at approximately 10°C, but a large proportion of the lipid adopted non-bilayer phases at all temperatures examined. Increasing the size of the headgroup by using poly-(ethyleneglycol)₅₀₀₀ conjugated to DOPE (DOPE-PEG₅₀₀₀) in place of DOPE-PEG₂₀₀₀, at the same molar fraction, caused a marked increase in bilayer stability. The bilayer to H_n transition temperature increased to approximately 30°C and the isotropic signal was barely discernible. The broadening

of the bilayer to H_{II} transition noted above is particularly evident here with H_{II} phase lipid present at 0°C and bilayer phase lipid present at 40°C.

D. *The influence of acyl chain composition on the bilayer stabilizing properties of BSCs*

The bilayer stabilizing ability of three BSCs differing only in acyl chain composition is shown in Figure 4. PEG₂₀₀₀ conjugated to dimyristoylphosphatidylethanolamine (DMPE-PEG₂₀₀₀), dipalmitoylphosphatidylethanolamine (DPPE-PEG₂₀₀₀) or distearoylphosphatidylethanolamine (DSPE-PEG₂₀₀₀) showed a similar ability to stabilize an equimolar mixture of DOPE and cholesterol. The bilayer to H_{II} phase transition was raised to approximately 40-50°C. The results are similar to those presented in Figure 2 which were obtained using a BSC with the same headgroup, but unsaturated acyl groups (DOPE-PEG₂₀₀₀) at the same concentration. The size of the isotropic signal varied somewhat with the different BSCs, being smallest with DSPE-PEG₂₀₀₀ and largest with DOPE-PEG₂₀₀₀ (cf., Figure 2 and Figure 4).

E. *The use of PEG-ceramides as bilayer stabilizing components*

The spectra set forth in Figures 1-4 were all obtained using PEG conjugated to phosphatidylethanolamine through a carbamate linkage. In addition, however, the use of ceramide as an alternative anchor for the hydrophilic polymer was examined. PEG₂₀₀₀ was conjugated via a succinate linker to egg ceramide. Figure 5 shows the ³¹P-NMR spectra obtained using mixtures of DOPE:cholesterol:egg ceramide-PEG₂₀₀₀ (1:1:0.1 and 1:1:0.25) over the temperature range of 0 to 60°C. At the lower molar ratio of PEG-ceramide, both bilayer and H_{II} phase lipid are in evidence at most temperatures. However, at the higher PEG-ceramide molar ratio, the spectra are exclusively bilayer up to 60°C at which point a low field shoulder corresponding to H_{II} phase lipid is visible. Unlike the spectra obtained using PEG-PEs, there was almost no isotropic signal when PEG-ceramide was used.

F. *Freeze-fracture electron microscopy*

One of the interesting features of several of the NMR spectra was the narrow signal at 0 ppm, indicative of isotropic motional averaging. This signal can arise

from a number of phospholipid phases such as micellar, small vesicular, cubic and rhombic phase structures. Freeze-fracture electron microscopy was used to investigate this aspect further. Figure 6 shows an electron micrograph of MLVs prepared by hydrating a mixture of DOPE:cholesterol:DOPE-PEG₂₀₀₀ (1:1:0.1) with HBS at room temperature. This lipid mixture corresponds to the NMR spectra set forth in Figure 2A which exhibited evidence of bilayer, H_n and isotropic phases.

A number of different structures are visible in the micrograph. Much of the lipid is present as large spherical vesicles of 400 to 600 nm in diameter. Many of the vesicles have indentations which appear to be randomly distributed in some vesicles, but organized in straight or curved lines in others. Cusp-like protrusions are also visible on the concave surfaces of some vesicles. These features are commonly referred to as lipidic particles (Verkleij, A.J., *Biochim. Biophys. Acta*, 779:43-92 (1984)) and may represent an intermediate structure formed during fusion of bilayers. These large vesicles would be expected to give rise to a predominately bilayer ³¹P-NMR spectrum with a narrow isotropic signal due to the lipidic particles. Similar results have been observed with *N*-methylated PEs (Gagne, *et al.*, *Biochemistry*, 24:4400-4408 (1985)). A number of smaller vesicles of around 100 nm diameter can also be seen. These vesicles may have been formed spontaneously on hydration, or may have been produced by vesiculization of larger vesicles. These vesicles are sufficiently small for lipid lateral diffusion, or tumbling of the vesicles in suspension, to produce motional averaging on the NMR timescale (Burnell, *et al.*, *Biochim. Biophys. Acta*, 603:63-69 (1980)), giving rise to an isotropic signal (see, Figure 2A). In the center of Figure 6 is a large aggregate showing evidence of several different structures. The right side of the aggregate is characterized by what appears to be closely packed lipidic particles. The upper left hand side shows a distinct organization into three-dimensional cubic arrays and the lower left hand region has the appearance of stacked tubes characteristic of lipid adopting the H_n phase (Hope, *et al.*, *J. Elect. Microsc. Tech.*, 13:277-287 (1989)). This is consistent with the corresponding ³¹P-NMR spectrum.

Figure 7 shows the appearance of the same mixture after extrusion through polycarbonate filters of 100 nm pore size to produce LUVS. The lipid is predominately organized into vesicles of approximately 100nm in diameter. Closer inspection reveals the presence of occasional larger vesicles and some of tubular shape. Overall the fairly

uniform size distribution is typical of that obtained when liposomes are produced by extrusion.

The presence of lipid micelles is not readily apparent from freeze fracture electron microscopy. Lipid in the micellar phase could, however, contribute to the isotropic signal observed in NMR spectra, and it has previously been shown that PEG-PE conjugates form micelles when hydrated in isolation (Woodle and Lasic, *Biochim. Biophys. Acta*, 113:171-199 (1992)). As such, the presence of micelles was tested by subjecting a suspension of LUVs to molecular sieve chromatography on Sepharose 4B. The liposomes were of the same composition used for the freeze fracture studies above except that DSPE-PEG₂₀₀₀ was used in place of DOPE-PEG₂₀₀₀, and they contained trace amounts of ¹⁴C-DPPC and ³H-DSPE-PEG₂₀₀₀. The elution profile is shown in Figure 8. A single peak containing both the phospholipid and PEG-PE conjugate markers was found in the void volume. A control experiment also shown in Figure 8 demonstrated that micelles, which formed when PEG-PE was hydrated in isolation, were included into the column and would have been clearly resolved if present in the liposomal preparation.

G. Effect of PE-PEG₂₀₀₀ On Fusion Of PE:PS LUVs

When unlabelled LUVs composed of DOPE:POPS (1:1) were added to fluorescently labelled LUVs there was a small jump in fluorescence intensity due to increased light scattering but no fusion (Figure 9, trace a). Upon addition of 5 mM Ca²⁺, there was a rapid increase in fluorescence consistent with lipid mixing as a result of membrane fusion. Fusion was complete within a few seconds and was followed by a slow decrease in fluorescence. Inspection of the cuvette revealed the presence of visible aggregates that settled despite stirring, resulting in the observed decrease in fluorescence. When PEG₂₀₀₀ conjugated to dimyristoylphosphatidylethanolamine (DMPE-PEG₂₀₀₀) was included in both vesicle populations, however, inhibition of fusion was observed. As shown in Figure 9 (traces b-d), inhibition was dependent on the concentration of DMPE-PEG₂₀₀₀ in the vesicles with as little as 2 mol% being sufficient to eliminate Ca²⁺ - induced fusion.

H. *The effect of PE-PEG loss on fusion*

Recently, it has been demonstrated that phospholipids conjugated to PEG of molecular weights 750-5,000 Da have enhanced rates of spontaneous transfer between liposomes. The half-time for transfer of these conjugates can vary from minutes to hours and depends on both the size of the PEG group and the nature of the acyl chains which anchor the conjugate in the bilayer. As such, fusion was examined under conditions where the PEG-lipid would be expected to transfer out of the liposomes. Ca^{2+} ions were added to PE:PS liposomes containing 2 mol% DMPE-PEG₂₀₀₀, followed by a twelve-fold excess (over labelled vesicles) of 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) liposomes as a sink for the PEG-PE. As shown in Figure 10 (curve a), while fusion was initially blocked by the presence of DMPE-PEG₂₀₀₀, the addition of POPC liposomes, which acted as a sink, lead to recovery of full fusogenic activity following a short time lag. The small initial jump in fluorescence intensity that occurred when POPC liposomes were added to PE:PS liposomes resulted from increased light scattering, not fusion. Control experiments demonstrated that no fusion occurred between the PE:PS liposomes and the POPC liposomes (data not shown), and no fusion occurred in the absence of POPC liposomes (Figure 10, curve b).

To confirm that recovery of fusogenic activity was dependent on removal of the PEG-PE, the influence of initial PEG-lipid concentration on the duration of the lag phase prior to fusion was examined (Figure 11). Liposomes containing equimolar PE and PS were prepared with 2, 3, 5 or 10 mol% DMPE-PEG₂₀₀₀. Fluorescently labelled and unlabelled vesicles were mixed at a ratio of 1:3 and after the addition of 5 mM CaCl_2 , a 36-fold excess (over labelled vesicles) of POPC liposomes was added. As expected, there was an increase in the time delay prior to fusion with increasing PEG-lipid concentration.

I. *The effect of conjugate acyl chain composition on fusogenic activity*

Since fusion is dependent on prior transfer of the PEG-PE out of the liposomes, it was thought that the rate at which fusogenic activity was recovered would depend on the rate of transfer of the PEG-PE. One factor that affects the rate at which a phospholipid transfers from one membrane to another is the length of its acyl chains. As such, the effect of conjugate acyl chain composition on fusogenic activity was

investigated. In doing so, the recovery of fusogenic activity of PE:PS LUVs containing 2 mol% DMPE-PEG₂₀₀₀ was compared with PE:PS LUVs containing 2 mol% DPPE-PEG₂₀₀₀ and 2 mol% DSPE-PEG₂₀₀₀ (Figure 12A). Increasing the length of the acyl chains from 14 to 16 carbons caused a dramatic increase in the lag period before fusion was initiated. Although the same level of fusion occurred using either DMPE-PEG₂₀₀₀ or DPPE-PEG₂₀₀₀, it was essentially complete in 40 minutes when DMPE-PEG₂₀₀₀ was the stabilizer, but took 24 hours when DPPE-PEG₂₀₀₀ was used. The implied decrease in rate of transfer (30–40 fold) is consistent with previous measurements of the effect of acyl chain length on rates of spontaneous phospholipid transfer. Increasing the acyl chain length to 18 carbons (DSPE-PEG₂₀₀₀, Figure 12A) extended the lag in fusion even further and, after 24 hours, the level was only 20% of maximum.

A second factor that affects the rate of spontaneous transfer of phospholipids between bilayers is the degree of saturation or unsaturation of the acyl chains. The rate of fusion of LUVs containing 2 mol% DOPE-PEG₂₀₀₀ is shown in Figure 12B. The presence of a double bond increased the rate of recovery of fusogenic activity in the presence of a sink for the DOPE-PEG₂₀₀₀ over that of the corresponding saturated species (DSPE-PEG₂₀₀₀, Figure 12A). The rate of fusion was similar to that seen with DPPE-PEG₂₀₀₀. Figure 12B also shows the rate of fusion obtained when the neutral PEG-lipid species, egg ceramide-PEG₂₀₀₀ was used. The rate was somewhat faster than observed with DPPE-PEG₂₀₀₀. Although differences in the interaction of the two lipid anchors with neighboring phospholipids in the bilayer make direct comparison of interbilayer transfer rates and, hence, fusion difficult, it appears that the presence of a negative charge on the conjugate (PE-PEG) is not required for desorption of the conjugate from negatively charged bilayers.

J. Effect of PEG molecular weight on fusogenic activity

The presence of PEG conjugated to the liposome surface results in a steric barrier that inhibits close bilayer apposition and subsequent fusion. The magnitude of the barrier should increase with increasing PEG molecular weight. When DMPE-PEG₃₀₀₀ was incorporated into PE:PS (1:1) LUVs, a concentration dependent inhibition of fusion was observed (Figure 13A). The results are similar to those obtained with DMPE-PEG₂₀₀₀ (Figure 9), except that only 1 mol% DMPE-PEG₃₀₀₀ was required to completely inhibit fusion compared to 2 mol% DMPE-PEG₂₀₀₀.

Figure 13B shows the effect of varying acyl chain composition of the larger PEG-lipid conjugate on fusion. Interestingly, the rates of fusion observed with 1 mol% PE-PEG₃₀₀₀ were similar to those with 2 mol% PE-PEG₂₀₀₀. The concentrations used were those shown to be sufficient to completely inhibit fusion (*cf.*, Figure 9 and Figure 13A). It was thought that the larger PEG group would increase the rate of interbilayer transfer of the conjugate and, hence, the rate of fusion. However, this was not the case. To examine this aspect further, the rates of fusion under conditions where the initial surface density of ethylene glycol groups was similar were compared. Figure 14 shows the fusion of PE:PS (1:1) LUVs containing 5 mol% DMPE-PEG₂₀₀₀ or 2 mol% DMPE-PEG₃₀₀₀ after addition of a sink for the PEG-lipid. The rates observed were very similar suggesting that factors other than loss of the steric barrier as a direct result of interbilayer transfer of the conjugate were involved.

K. Programmable fusogenic liposomes comprising DOPE:cholesterol:DODAC:ceramides

Fluorescently labelled liposomes were prepared in distilled water from a mixture of DOPE and *N,N*-dioleoyl-*N,N*-dimethylammonium chloride (DODAC) at a molar ratio of 85:15. A three-fold excess of acceptor liposomes of the same composition, but containing no fluorescent probes, was added to labelled liposomes and fusion was initiated after 60s by the addition of NaCl (Figure 15). Fusion was highly dependent on ionic strength. Little fusion was observed at 50 mM NaCl, but with increasing salt concentration, the rate and extent of fusion increased dramatically. At 300 mM NaCl fusion was so extensive that visible aggregates occurred and these aggregates could not be maintained in suspension resulting in the apparent decrease in fluorescence seen in Figure 15 for the 300 mM NaCl curve. Importantly, substantial fusion was observed at physiological salt concentration (150 mM).

As described above, the inclusion of 2 mol% PEG-lipid in PE:PS liposomes is sufficient to inhibit Ca²⁺-induced fusion. When 2 mol% DMPE-PEG₂₀₀₀ was included in DOPE:DODAC liposomes (DOPE:DODAC:DMPE-PEG₂₀₀₀, 83:15:2), the same inhibitory effect was observed (Figure 16). However, unlike the PE:PS system, when these liposomes were incubated for 1hr in the presence of a large excess of POPC liposomes, which acted as a sink for the PEG-PE, little, if any, fusion was observed. Since PEG-PEs are negatively charged the complementary charge, interaction

with DODAC likely results in a dramatic decrease in the rate of transfer out of the bilayer.

As an alternative bilayer stabilizing component, therefore, the ability of a neutral PEG-lipid species, *i.e.*, PEG-ceramide, to inhibit fusion in this system was examined. PEG-ceramides have similar bilayer stabilizing properties to PEG-PEs. For these studies, PEG₂₀₀₀ was conjugated to ceramides of various fatty amide chain lengths through a succinate linker. Liposomes prepared from DOPE:DODAC:(C8:0) ceramide-PEG₂₀₀₀ (83:15:2) did not fuse in the presence of 300 mM NaCl. However, when an excess of POPC liposomes was added, fusion occurred fairly rapidly (Figure 17).

Similar results were observed when cholesterol was incorporated into the liposomes (DOPE:cholesterol:DODAC:(C8:0) ceramide-PEG₂₀₀₀, 38:45:15:2), although the rate of fusion was slower than with cholesterol-free liposomes (Figure 17).

To determine if the rate of fusion in this system can be controlled, the chain lengths of the fatty amide groups of the PEG-ceramides were varied. Using a (C14:0) ceramide-PEG₂₀₀₀, 50% maximal fusion was observed after approximately 6 hr (Figure 18). This was a dramatic increase over the rate with (C8:0) ceramide-PEG₂₀₀₀ shown in Figure 18, where maximal fusion was achieved in about 40 minutes. The time for 50% maximal fusion was increased to over 20 hr when egg ceramide-PEG₂₀₀₀ was used. Ceramides derived from egg have a fatty amide chain length of predominantly 16:0 (approximately 78%), with small amounts of longer saturated chains. Figure 18 also shows an extended time course with DMPE-PEG₂₀₀₀. The limited extent of fusion (<20% of maximum at 21 hr) shows the dramatic effect that charge interaction can have on PEG-lipid transfer rates.

The rationale for using cationic liposomes is that complementary charge interaction with anionic plasma membranes will promote association and fusion of liposomes with cells *in vivo*. It is important, therefore, to confirm that not only will DOPE:DODAC liposomes fuse with membranes carrying a negative charge, but that incorporation of PEG-lipid conjugates prevents fusion in a programmable manner. This ability is demonstrated in Figure 19 which shows that liposomes composed of DOPE:cholesterol:DODAC, 40:45:15, fuse with negatively charged liposomes and inclusion of a PEG-lipid conjugate in the cationic liposomes inhibits fusion. Fusion between DOPE:DODAC liposomes could be prevented when 2 mol% PEG-lipid was present in both fluorescently labelled and acceptor liposomes. When PEG-lipid was

omitted from the acceptor liposomes, however, its concentration in the labelled vesicles had to be increased to 4-5 mol% to block fusion between cationic and anionic liposomes.

Again, while PEG-lipids can inhibit fusion in this system, under conditions where the PEG-lipid can transfer out of the liposomes, fusogenic activity can be restored.

5 Figure 20 shows that this is, indeed, the case. Incubation of DOPE:cholesterol:DODAC:(C14:0) ceramide-PEG₂₀₀₀ (36:45:15:4) liposomes with PE:PS liposomes, in the presence of excess POPC:cholesterol (55:45) vesicles which act as a sink, results in recovery of fusogenic activity. In the absence of a sink, a slow rate of fusion is observed, indicating that a higher concentration of PEG-lipid is required to completely
10 prevent fusion over longer periods.

While fusion between cationic and anionic liposomes provides a good model system, fusion *in vivo* is somewhat different. The acceptor membrane is not composed solely of lipid, but contains a high concentration of proteins, many of which extend outward from the lipid bilayer and may interfere with fusion. Using erythrocyte
15 ghosts as a representative membrane system, it was found that liposomes composed of DOPE:cholesterol:DODAC (40:45:15) fuse with cellular membranes (*see*, Figure 21). In addition, it was found that fusion in this system, like those presented above, can also be inhibited using PEG-lipid conjugates. This results clearly establish the usefulness of these systems as programmable fusogenic carriers for intracellular drug delivery.

20

L. Programmed fusion with Erythrocytes (RBCs)

LUVs composed of DOPE:cholesterol:DODAC (40:45:15) fused rapidly and extensively with RBCs (Figure 22, panels a and b). Prolonged incubation caused extensive lysis of the RBCs and numerous fluorescently labeled "ghosts" were formed.
25 Incorporation of PEG-ceramide (C8:0) at 5 mol% blocked fusion (Figure 22, panels c and d) and this effect was maintained for up to 24 hr. This effect was somewhat surprising since the (C8:0) ceramide can exchange rapidly (*i.e.*, within minutes) between liposomal membranes. It appears that either the RBCs cannot act as a sink for the PEG-ceramide, or there were insufficient RBCs to remove enough PEG-ceramide to allow
30 fusion. However, when an exogenous sink for the PEG-ceramide was included, fusogenic activity was recovered within minutes (Figure 22, panels e and f).

When PEG-ceramides with longer fatty amide chains (*i.e.*, C14:0 or C20:0) were used, there was little fusion over 24 hr, even in the presence of an

exogenous sink. This again was surprising as substantial fusion is observed over this time frame in liposomal systems when a sink is present. It was thought that some non-specific interaction between the sink (POPC/cholesterol) and the RBCs was occurring which hindered the ability of the POPC:cholesterol liposomes to absorb the PEG-ceramide. To overcome this, the fusogenic liposomes were pre-incubated with the sink before adding RBCs. Figure 23 shows the results obtained under these conditions using PEG-ceramide (C14:0). No fusion was observed after pre-incubation of the fusogenic LUVs with the sink for 5 minutes prior to addition of RBCs (Figure 23, panels a and b). However, after a 1 hr pre-incubation, some fusion with RBCs was observed (Figure 23, panels c and d), suggesting that under these conditions the PEG-ceramide could transfer out of the liposomes and became fusogenic. With longer incubations (2hr), the pattern of fluorescent labeling changed. Rather than diffuse labeling of the RBC plasma membranes, extensive punctate fluorescence was observed (Figure 23, panels e and f) and this pattern was maintained for up to 24 hr. The punctate fluorescence did not appear to be associated with cells and it may represent fusion of fluorescent liposomes with the sink, although previous fluorescent measurements of liposome-liposome fusion indicated that this did not occur to any appreciable extent. A second possibility is that exchange of the fluorescent probe over the longer time courses leads to labeling of the sink, although it seems unlikely that this would prevent fusion and labeling of the RBCs. When PEG-ceramide (C20:0) was used, there was no evidence for fusion after preincubation of LUVs with the sink for 5 min (Figure 24, panels a and b), 1 hr (Figure 24, panels c and d), 2 hr (Figure 24, panels e and f), or for up to 24 hr (results not shown).

Figures 22-24 unequivocally establish that the liposomes of the present invention exhibit programmable fusion with intact cells. Firstly, liposomes composed of DOPE:cholesterol:DODAC (40:45:15) that contain no PEG-lipid fuse rapidly and extensively with RBCs. Secondly, when the liposomes contain 5 mol% PEG-lipid fusion is blocked regardless of the composition of the lipid anchor. Thirdly, in the presence of a sink to which the PEG-lipid can transfer, fusogenic activity can be restored at a rate that is dependent on the nature of the lipid anchor. Although exchange leading to fusion could not be demonstrated when the PEG-ceramide (C20:0) was used, it is believed this is a problem with the assay rather than a lack of fusogenic potential. *In vivo* there would be an almost infinite sink for PEG-lipid exchange.

M. Inhibition of transmembrane carrier system (TCS) fusion by PEG₂₀₀₀-Ceramide (C14:0) and PEG₂₀₀₀-DMPE

TCS composed of 1,2-dioleoyl-3-phosphatidylethanolamine (DOPE), N,N-dioleoyl-N,N-dimethylammoniumchloride (DODAC), the fluorophores N-(7-nitro-2-1,3-benzoxadiazol-4-yl)-1,2-dioleoyl-sn-phosphatidylethanolamine (NBD-PE) and N-(lissamine rhodamine B sulfonyl)-1,2-dioleoyl-sn-phosphatidylethanolamine (Rh-PE), and either PEG₂₀₀₀-Ceramide (C14:0) or PEG₂₀₀₀-DMPE were prepared by extrusion through 100 nm diameter polycarbonate filters (Hope, M.J., *et al.*, *P.R. Biochim. Biophys. Acta.* 812, 55-65 (1985)). TCS contained 0.5 mol% NBD-PE and 0.5 mol% Rh-PE and either DOPE:DODAC:PEG₂₀₀₀-DMPE (80:15:5 mol%) or DOPE:DODAC:PEG₂₀₀₀-Ceramide (C14:0) (80:15:5 mol%). Fluorescently labelled liposomes were incubated at 37°C in 20 mM HEPES, 150 mM NaCl, pH 7.4 (HBS) with a three-fold excess of liposomes composed of DOPE:POPS (85:15 mol%). POPS liposomes were added at 10-fold the concentration of the fluorescently labelled liposomes and lipid mixing was assayed by the method of Struck, D.K., *et al.* (*Biochemistry* 20, 4093-4099 (1981)). The excitation wavelength used was 465 nm and an emission filter placed at 530 nm minimized intensity due to scattered light. Rates and extents of fusion were followed by monitoring the increase in NBD fluorescence intensity at a wavelength of 535 nm over time. Percent maximum fusion was determined from the relationship $\text{Fusion (\% max)}(t) = (F(t) - F_0) / (F_{\infty} - F_0)$, where F_0 is the initial NBD fluorescence intensity at time zero, $F(t)$ is the intensity at time t and F_{∞} is the maximum achievable fluorescence intensity under conditions of complete lipid mixing of fluorescently labelled and DOPC:POPS liposomes (Bailey, A.L., *et al.*, *P.R. Biochemistry* 33, 12573-12580 (1994)). Figure 25 illustrates considerable mixing of DOPE/DODAC/PEG₂₀₀₀-Ceramide (C14:0) with DOPC:POPS compared to that of DOPE/DODAC/PEG₂₀₀₀-DMPE with DOPC:POPS, suggesting that the PEG₂₀₀₀-DMPE is only minimally removed from the TCS. This result is attributed to the electrostatic interaction between the anionic PEG₂₀₀₀-DMPE and cationic DODAC which effectively decreases the monomer concentration of the PEG₂₀₀₀-DMPE in aqueous solution.

N. *In vivo stabilization of liposomes containing cationic lipids using amphiphatic bilayer stabilizing components*

The ability of a series of bilayer stabilizing components (e.g., PEG-modified lipids) to stabilize fusogenic liposomes containing a cationic lipid *in vivo* were examined in this study. A freeze-fracture electron microscope analysis of liposomes composed of dioleoylphosphatidylethanolamine (DOPE) and N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC) showed that inclusion of a bilayer stabilizing component, e.g., PEG-DSPE and PEG-Ceramide, effectively prevented liposome aggregation in the presence of mouse serum. Biodistribution of fusogenic liposomes composed of DOPE and DODAC, additionally containing a bilayer stabilizing component (i.e., an amphiphatic polyethyleneglycol (PEG) derivative), were then examined in mice using ³H-labelled cholesterylhexadecylether as a lipid marker. Bilayer stabilizing components included PEG-DSPE and various PEG-Ceramide (PEG-Cer) with different acyl chain length ranging from C8 to C24. DOPE/DODAC liposomes (85:15, mol/mol) were shown to be cleared rapidly from the blood and accumulate exclusively in the liver. Inclusion of a bilayer stabilizing component at 5.0 mol% of the lipid mixture resulted in increased liposome levels remaining in the blood and concomitantly decreased accumulation in the liver. Among the various bilayer stabilizing components, PEG-DSPE shows the highest activity in prolonging the circulation time of DOPE/DODAC liposomes. The activity of PEG-Ceramide is directly proportional to the acyl chain length: the longer the acyl chain, the higher the activity. The activity of PEG-Ceramide (C20) exhibiting the optimal acyl chain length depends on its concentration of the lipid mixture, with the maximal circulation time obtained at 30 mol% of the lipid mixture. While inclusion of bilayer stabilizing components in the lipid composition generally results in increased circulation time of DOPE/DODAC liposomes, the presence of a cationic lipid, DODAC, appeared to promote their rapid clearance from the blood.

The preparations and uses of DODAC liposomes are disclosed in U.S. Patent Application Number 08/316,399, filed September 30, 1994, the teachings of which are incorporated herein by reference.

1. Materials and Methods

a. Liposome Preparation

Small unilamellar liposomes composed of DOPE and DODAC and bilayer stabilizing components at various ratios were prepared by the extrusion method. Briefly, the solvent-free lipid mixture containing ³H-labelled CHE, as a nonexchangeable and nonmetabolizable lipid marker, was hydrated with distilled water overnight. Normally, the liposome suspension (5 mg lipid per ml) was extruded, at room temperature, 10 times through stacked Nuclepore membranes (0.1 µm pore size) using an extrusion device obtained from Lipex Biomembranes, Inc. to generate liposomes with homogeneous size distributions. Liposome size was determined by quasi-elastic light scattering using a particle sizer and expressed as average diameter with standard deviation (SD).

b. Liposome Biodistribution Study

³H-labelled liposomes with various lipid compositions were injected i.v. into female CD-1 mice (8-10 weeks old) at a dose of 1.0 mg lipid per mouse in 0.2 ml of distilled water. At specified time intervals, mice were killed by overexposure to carbon dioxide, and blood was collected via cardiac puncture in 1.5-ml microcentrifuge tubes and centrifuged (12000 rpm, 2 min, 4°C) to pellet blood cells. Major organs, including the spleen, liver, lung, heart, and kidney, were collected, weighed, and homogenized in distilled water. Fractions of the plasma and tissue homogenates were transferred to glass scintillation vials, solubilized with Solvable (NEN) at 50°C according to the manufacturer's instructions, decolorized with hydrogen peroxide, and analyzed for ³H radioactivity in scintillation fluid in a Beckman counter. Data were expressed as percentages of the total injected dose of ³H-labelled liposomes in each organ. Levels of liposomes in the plasma were determined by assuming that the plasma volume of a mouse is 5.0% of the total body weight.

2. Results and Discussion

a. Freeze-Fracture Electron Microscopic Studies

Liposomes composed of DOPE/DODAC (85:15, mol/mol), DOPE/DODAC/PEG-Ceramide (C20) (80:15:5, mol/mol), and DOPE/DODAC/PEG-DSPE (80:15:5, mol/mol) were prepared by the extrusion method and had similar average diameters (100 nm). Freeze-fracture electron micrographs of the three liposomal

formulations showed unilamellar liposomes with relatively narrow size ranges.

However, preincubation of DOPE/DODAC liposomes in 50% mouse serum at 37°C for 30 minutes resulted in their massive aggregations. On the other hand, both DOPE/DODAC/PEG-Ceramide (C20) and DOPE/DODAC/PEG-DSPE liposomes did not show any aggregation when these liposomes were pretreated with mouse serum. Thus, these results show the effectiveness of the bilayer stabilizing components in preventing serum-induced rapid aggregations of DOPE/DODAC liposomes.

b. Biodistribution of DOPE/DODAC Liposomes Containing Bilayer Stabilizing Components, *i.e.*, Amphiphatic PEG Derivatives

DOPE/DODAC liposomes with or without bilayer stabilizing components were prepared to include ³H-labelled cholesterolhexadecylether as a lipid marker, and their biodistribution was examined in mice at 1 hour after injection. Liposomes tested in this study were composed of DOPE/DODAC (85:15, mol/mol), DOPE/DODAC/PEG-Ceramide (80:15:5, mol/mol), and DOPE/DODAC/PEG-DSPE (80:15:5, mol/mol). To also examine the effect of the hydrophobic anchor on biodistribution of liposomes, various PEG-Ceramide derivatives with different acyl chain lengths were used. These liposomal formulations had similar average diameters, ranging from 89 to 103 nm. Table I below shows levels of liposomes in the blood, spleen, liver, lung, heart, and kidney, together with respective blood/liver ratios. DOPE/DODAC liposomes were shown to be cleared rapidly from the blood and accumulate predominantly in the liver with the blood/liver ratio of approximately 0.01. Inclusion of bilayer stabilizing components at 5.0 mol% in the lipid composition resulted in their increased blood levels and accordingly decreased liver accumulation to different degrees.

DOPE/DODAC/PEG-DSPE liposomes showed the highest blood level (about 59%) and the lowest liver accumulation (about 35%) with the blood/liver ratio of approximately 1.7 at 1 hour after injection. Among various PEG-Ceramide derivatives with different acyl chain lengths, PEG-Ceramide (C20)-containing liposomes showed the highest blood level (about 30%) with the blood/liver ratio of approximately 0.58, while PEG-Ceramide (C8)-containing liposomes showed a lower blood level (about 6%) with the blood/liver ratio of approximately 0.1. It appeared that, among different PEG-Ceramide derivatives, the activity in increasing the blood level of liposomes is directly proportional to the acyl chain length of ceramide; the longer the acyl chain length, the greater the activity. It

also appeared that the optimal derivative for increasing the blood level of liposomes is PEG-Ceramide (C20).

c. Optimization of DOPE/DODAC Liposomes for Prolonged Circulation Times

The effect of increasing concentrations of PEG-Ceramide (C20) in the lipid composition on biodistribution of DOPE/DODAC liposomes was examined. PEG-Ceramide (C20) was included in DOPE/DODAC liposomes at increasing concentrations (0-30 mol%) in the lipid composition, while the concentration of DODAC was kept at 15 mol% of the lipid mixture. Liposomes were prepared by the extrusion method and had similar average diameters ranging from 102 nm to 114 nm. Liposomes were injected i.v. into mice, and biodistribution was examined at 1 hour after injections. Figure 26 shows the liposome level in the blood and liver at 1 hour after injections as a function of the PEG-Ceramide (C20) concentration. Clearly, increasing the concentration of PEG-Ceramide in the lipid composition resulted in progressive increase in liposome levels in the blood, accompanied by decreased accumulation in the liver. The highest blood level (about 84 % at 1 hour after injection) was obtained for DOPE/DODAC/PEG-Ceramide (C20) (55:15:30, mol/mol) showing the blood/liver ratio of about 6.5.

The effect of increasing concentrations of DODAC on the biodistribution of DOPE/DODAC liposomes also was examined. DOPE/DODAC liposomes containing either 10 mol% or 30 mol% PEG-Ceramide (C20) and various concentrations (15, 30, 50 mol%) were prepared by the extrusion method and had similar average diameters ranging from 103 to 114 nm. Biodistribution was examined at 1 hour after injections, and expressed as percentages of liposomes in the blood as a function of the DODAC concentration (Figure 27). As shown in Figure 27, increasing DODAC concentrations in the lipid composition resulted in decreased levels in the blood for both liposomal formulations. Thus, the presence of a cationic lipid, DODAC, in the lipid composition results in rapid clearance from the blood. Also, shown in Figure 27 is that such a DODAC effect can be counteracted by increasing the concentration of PEG-Ceramide (C20) in the lipid composition.

Figure 28 shows time-dependent clearances of DOPE/DODAC liposomes with or without PEG-Ceramide from the blood. Only a small fraction of injected DOPE/DODAC liposomes remained in the blood, while increasing the concentration of

PEG-Ceramide (C20) in the lipid composition resulted in prolonged circulation times in the blood. Estimated half-lives in the α -phase for DOPE/DODAC/PEG-Ceramide (C20) (75:15:10, mol/mol) and DOPE/DODAC/PEG-Ceramide (C20) (55:15:30, mol/mol) were < 1 hour and 5 hours, respectively.

5

3. Conclusions

The above studies indicate that there are several levels at which biodistribution of fusogenic liposomes containing a cationic lipid can be controlled by inclusion of bilayer stabilizing components. Data in Table I shows that the hydrophobic anchor of the bilayer stabilizing components has an important role in determining biodistribution of DOPE/DODAC liposomes. Studies using various PEG-Ceramide derivatives with different acyl chain lengths showed that the longer the acyl chain length of PEG-Ceramide, the greater the activity in prolonging the circulation time of DOPE/DODAC liposomes. These results are consistent with the rate at which the bilayer stabilizing components dissociate from the liposome membrane being directly proportional to the size of the hydrophobic anchor. Accordingly, PEG-Ceramide derivatives with a longer acyl chain can have stronger interactions with other acyl chains in the liposome membrane and exhibit a reduced rate of dissociation from the liposome membrane, resulting in stabilization of DOPE/DODAC liposomes for a prolonged period of time and thus their prolonged circulation time in the blood.

In addition to the hydrophobic anchor of the bilayer stabilizing components, the concentration of the bilayer stabilizing components in the lipid membrane can also be used to control *in vivo* behavior of DOPE/DODAC liposomes. Data in Figure 26 show that increasing the concentration of PEG-Ceramide (C20) in the lipid composition resulted in increased liposome levels in the blood. The optimal concentration of PEG-Ceramide (C20) in the lipid composition was found to be 30 mol% of the lipid mixture. It appeared that the circulation time of DOPE/DODAC/PEG-Ceramide (C20) liposomes is determined by the relative concentrations of two lipid compositions, DODAC and PEG-Ceramide, exhibiting opposite effects on liposome biodistribution. While bilayer stabilizing components exhibit the ability to prolong the circulation time of liposomes in the blood, a cationic lipid, DODAC, exhibits the ability to facilitate liposome clearance from the blood. Thus, for the maximal circulation time in the blood, an appropriate concentration of a bilayer stabilizing component and a

minimal concentration of DODAC should be used. It should be noted, however, that an optimal liposome formulation for the prolonged circulation time in the blood is not necessarily the one suitable for an intended application in delivery of certain therapeutic agents. Both pharmacokinetic and pharmacodynamic aspects of fusogenic liposomes should be examined for different applications using different therapeutic agents.

5

TABLE II: Effect of Amphipathic PEG Derivatives on Biodistribution of DOPE/DODAC Liposomes

5

5 initiated dose

PEG-Derivative	Average Diameter (nm)	Blood	Liver	Spleen	Lung	Heart	Kidney	Total	Blood/Liver
None	103 (29)	0.8 (0.4)	64.4 (2.0)	3.1 (1.8)	1.2 (0.2)	0.2 (0.0)	0.3 (0.0)	70.0 (1.4)	0.012
PEG-DSPE	95 (26)	59.1 (8.2)	34.7 (2.1)	2.9 (0.1)	1.9 (0.6)	1.7 (0.4)	1.2 (0.3)	101.4 (6.1)	1.703
PEG-Cer (C8)	89 (24)	6.5 (1.9)	62.8 (1.4)	4.2 (1.0)	0.5 (0.3)	0.3 (0.1)	0.3 (0.1)	74.6 (5.1)	0.104
PEG-Cer (C14)	93 (23)	5.9 (0.3)	55.9 (1.0)	3.3 (0.2)	0.1 (0.0)	0.1 (0.0)	0.1 (0.0)	65.4 (1.6)	0.106
PEG-Cer (C16)	93 (24)	13.9 (2.1)	57.5 (2.0)	2.6 (0.1)	0.0 (0.0)	0.2 (0.1)	0.0 (0.0)	74.3 (4.0)	0.242
PEG-Cer (C20)	101 (24)	29.8 (4.8)	51.0 (2.2)	1.9 (0.2)	0.0 (0.0)	0.3 (0.1)	0.0 (0.0)	82.8 (2.8)	0.514
PEG-Cer (C24)	92 (28)	26.7 (0.8)	46.7 (7.6)	5.7 (1.2)	1.0 (0.2)	0.9 (0.2)	0.4 (0.1)	81.5 (4.1)	0.572

³H-labelled liposomes composed of DOPE/DODAC (75:15, mol/mol) additionally containing an indicated PEG derivative at 5.0 mol% of the lipid mixture were injected i.v. into mice. Biodistribution was examined at 1 h after injection and expressed as percentage of injected dose of liposomes with SD (n=3).

15

The foregoing is offered for purposes of illustration. It will be readily apparent to those skilled in the art that the operating conditions, materials, procedural steps and other parameters of the methods and test devices described herein may be further modified or substituted in ways without departing from the spirit and scope of the invention.

5

WHAT IS CLAIMED IS:

1. A fusogenic liposome comprising:
a lipid capable of adopting a non-lamellar phase, yet capable of assuming a
bilayer structure in the presence of a bilayer stabilizing component;
and
5 a bilayer stabilizing component reversibly associated with said lipid to
stabilize said lipid in a bilayer structure.
2. The fusogenic liposome in accordance with claim 1 wherein said
lipid is a phosphatidylethanolamine.
3. The fusogenic liposome in accordance with claim 2 wherein said
lipid is an unsaturated phosphatidylethanolamine.
4. The fusogenic liposome in accordance with claim 1 wherein said
lipid is a mixture of a phosphatidylethanolamine and a phosphatidylserine.
5. The fusogenic liposome in accordance with claim 1 wherein said
lipid is a mixture of a phosphatidylethanolamine and a cationic lipid.
6. The fusogenic liposome in accordance with claim 5 wherein said
cationic lipid is a member selected from the group consisting of 3 β -(N-(N',N'-
dimethylaminoethane)carbamoyl)cholesterol, N,N-distearyl-N,N-dimethylammonium
bromide, N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium
bromide, diheptadecylamidoglycyl spermidine, N-(1-(2,3-dioleyloxy)propyl)-N-(2-
5 (sperminecarboxamido)ethyl)-N,N-dimethylammonium trifluoroacetate, N-(1-(2,3-
dioleyloxy)propyl)-N,N,N-trimethylammonium chloride, N-(1-(2,3-dioleyloxy)propyl)-
N,N,N-trimethylammonium chloride and N,N-dioleyl-N,N-dimethylammonium chloride.
7. The fusogenic liposome in accordance with claim 1 further
comprising cholesterol.

8. The fusogenic liposome in accordance with claim 1 wherein said bilayer stabilizing component is a member selected from the group consisting of lipids, lipid derivatives, detergents, proteins and peptides.

9. The fusogenic liposome in accordance with claim 1 wherein said bilayer stabilizing component is polyethyleneglycol coupled to a phosphatidylethanolamine.

10. The fusogenic liposome in accordance with claim 9 wherein said polyethyleneglycol has a molecular weight ranging from about 200 to 10,000.

11. The fusogenic liposome in accordance with claim 9 wherein said polyethyleneglycol has a molecular weight ranging from about 1,000 to 8,000.

12. The fusogenic liposome in accordance with claim 9 wherein said polyethyleneglycol has a molecular weight ranging from about 2,000 to 6,000.

13. The fusogenic liposome in accordance with claim 9 wherein said phosphatidylethanolamine is a member selected from the group consisting of dimyristoylphosphatidylethanolamine, dipalmitoylphosphatidylethanolamine, dioleoylphosphatidylethanolamine and distearoylphosphatidylethanolamine.

14. The fusogenic liposome in accordance with claim 1 wherein said bilayer stabilizing component is polyethyleneglycol coupled to a ceramide.

15. The fusogenic liposome in accordance with claim 1 wherein said bilayer stabilizing component is present at a concentration ranging from about 0.05 mole percent to about 50 mole percent.

16. The fusogenic liposome in accordance with claim 15 wherein said bilayer stabilizing component is present at a concentration ranging from about 0.05 mole percent to about 30 mole percent.

17. The fusogenic liposome in accordance with claim 15 wherein said bilayer stabilizing component is present at a concentration ranging from about 1 mole percent to about 20 mole percent.

18. The fusogenic liposome in accordance with claim 7 wherein said cholesterol is present at a concentration ranging from about 0.02 mole percent to about 50 mole percent.

19. The fusogenic liposome in accordance with claim 1 wherein the rate at which said liposome becomes fusogenic can be varied over a timescale ranging from minutes to days.

20. The fusogenic liposome in accordance with claim 1 wherein the rate at which said liposome becomes fusogenic can be controlled.

21. The fusogenic liposome in accordance with claim 20 wherein the rate at which said liposome becomes fusogenic is controlled by controlling the composition of said bilayer stabilizing component in said fusogenic liposome.

22. The fusogenic liposome in accordance with claim 20 wherein the rate at which said liposome becomes fusogenic is controlled by controlling the concentration of said bilayer stabilizing component in said fusogenic liposome.

23. A method for delivering a therapeutic compound to a target cell at a predetermined rate, comprising:

5 administering to a host containing said target cell a fusogenic liposome which comprises a bilayer stabilizing component, a lipid capable of adopting a non-lamellar phase, yet capable of assuming a bilayer structure in the presence of said bilayer stabilizing component, and said therapeutic compound or a pharmaceutically acceptable salt thereof.

24. The method in accordance with claim 23 wherein said bilayer stabilizing component is a member selected from the group consisting of lipids, lipid derivatives, detergents, proteins and peptides.

25. The method in accordance with claim 23 wherein said bilayer stabilizing component is polyethyleneglycol coupled to a phosphatidylethanolamine.

26. The method in accordance with claim 23 wherein said bilayer stabilizing component is polyethyleneglycol coupled to a ceramide.

27. The method in accordance with claim 23 wherein said lipid is a phosphatidylethanolamine.

28. The method in accordance with claim 23 wherein said lipid is a mixture of an phosphatidylethanolamine and a phosphatidylserine.

29. The method in accordance with claim 23 wherein said lipid is a mixture of a phosphatidylethanolamine and a cationic lipid.

30. The method in accordance with claim 23 wherein said fusogenic liposome further comprises cholesterol.

31. The method in accordance with claim 23 wherein said fusogenic liposome is administered intravenously.

32. The method in accordance with claim 23 wherein said fusogenic liposome is administered parenterally.

33. The method in accordance with claim 23 wherein said fusogenic liposome administered to said host is unilamellar.

34. The method in accordance with claim 33 wherein said unilamellar fusogenic liposome has a mean diameter of 0.05 microns to 0.45 microns.

35. The method in accordance with claim 34 wherein said unilamellar fusogenic liposome has a mean diameter of 0.05 microns to 0.2 microns.

36. A method of stabilizing in a bilayer structure a lipid which is capable of adopting a non-lamellar phase, said method comprising: combining a bilayer stabilizing component with a lipid which is capable of adopting a non-lamellar phase, yet which is capable of assuming a bilayer structure in the presence of said bilayer stabilizing component, said bilayer stabilizing component being selected to be exchangeable or biodegradable such that over a predetermined period of time, said bilayer stabilizing component is lost from said bilayer structure or modified, thereby rendering said bilayer structure fusogenic.

37. The method in accordance with claim 36 wherein said bilayer stabilizing component is a member selected from the group consisting of lipids, lipid derivatives, detergents, proteins and peptides.

38. The method in accordance with claim 36 wherein said bilayer stabilizing component is polyethyleneglycol coupled to a phosphatidylethanolamine.

39. The method in accordance with claim 36 wherein said bilayer stabilizer is polyethyleneglycol coupled to a ceramide.

40. The method in accordance with claim 36 wherein said lipid is a phosphatidylethanolamine.

41. The method in accordance with claim 36 wherein said lipid is a mixture of a phosphatidylethanolamine and a phosphatidylserine.

42. The method in accordance with claim 36 wherein said lipid is a mixture of a phosphatidylethanolamine and a cationic lipid.

43. The method in accordance with claim 36 further comprising cholesterol.

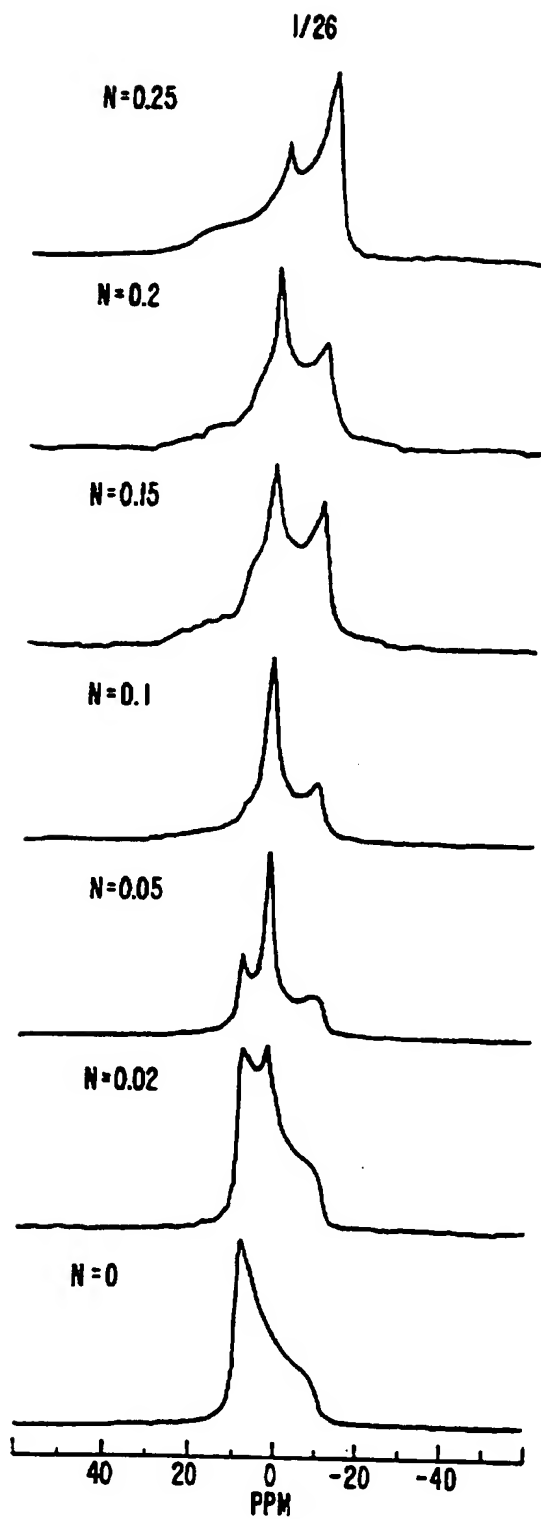
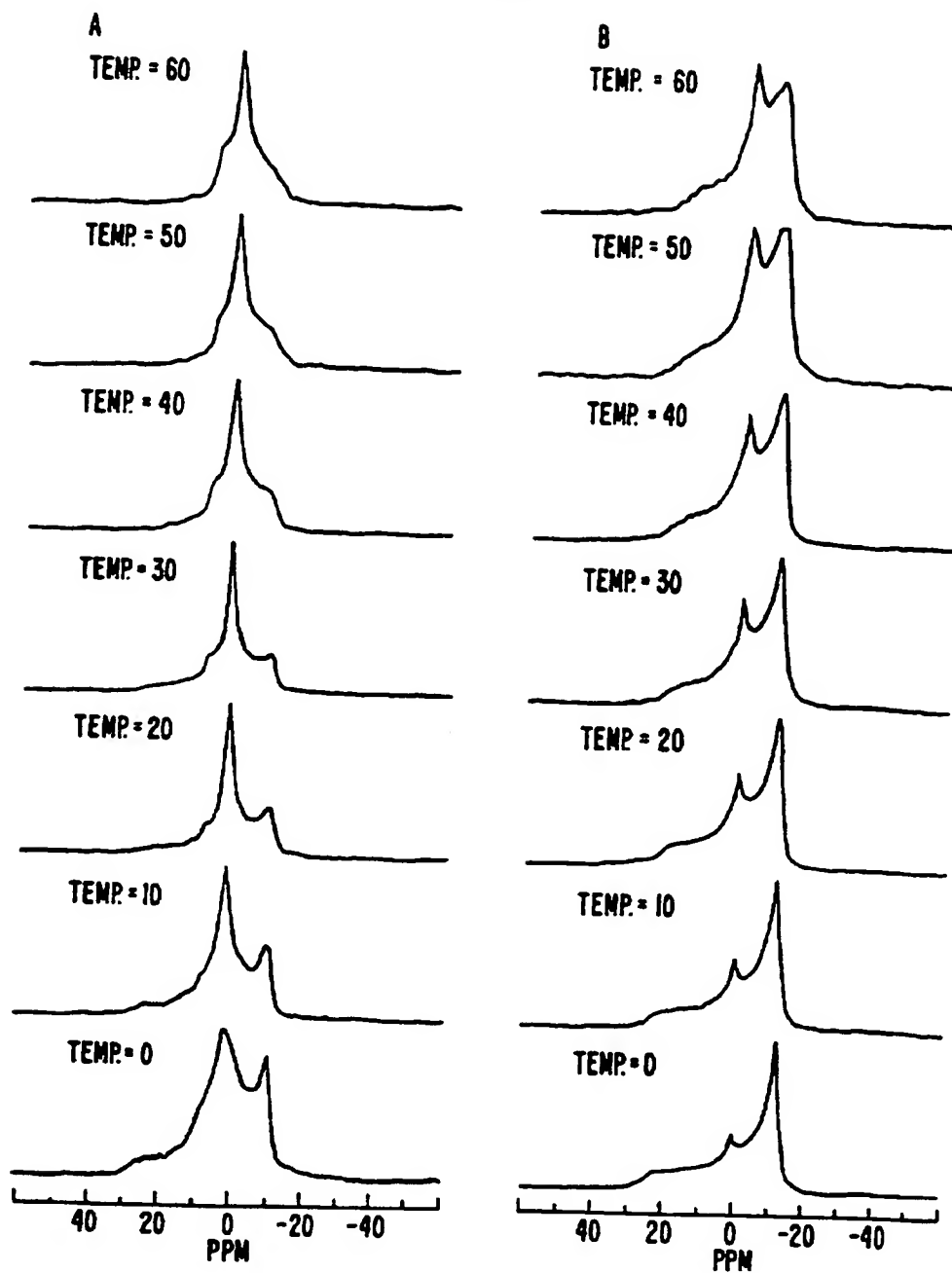
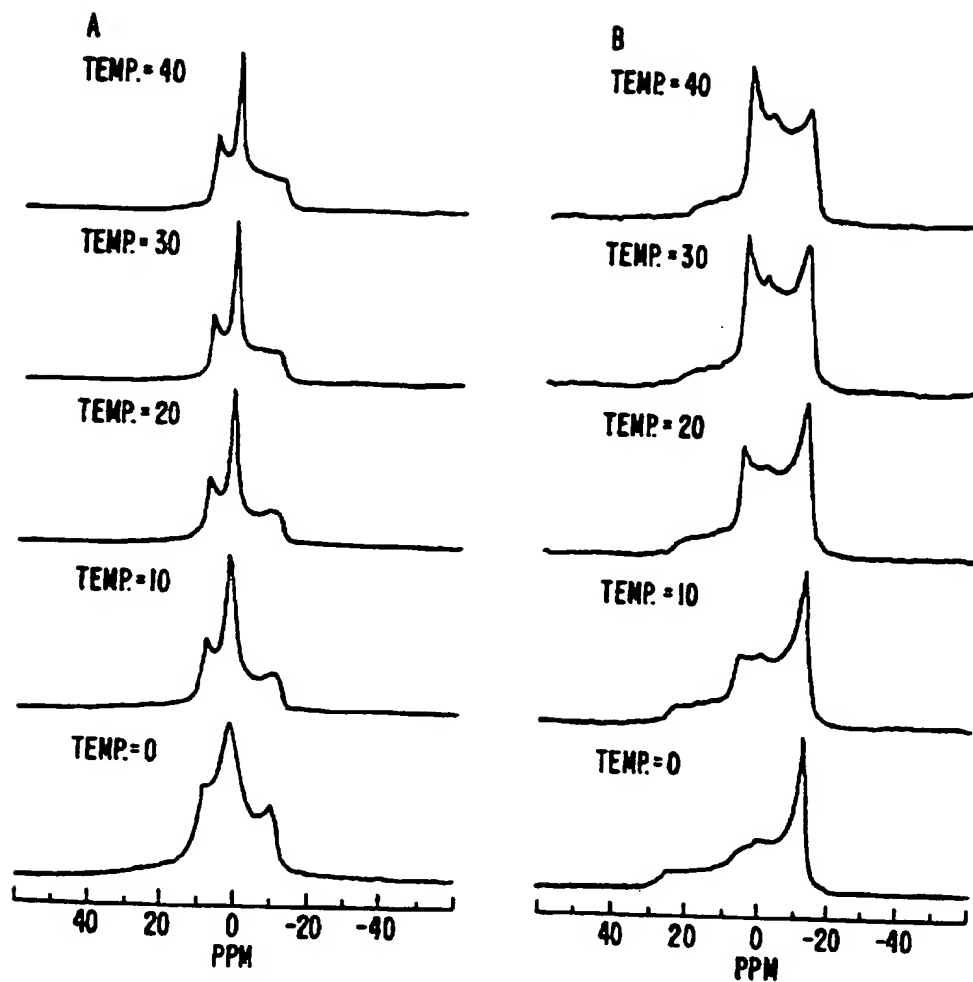


FIG. 1
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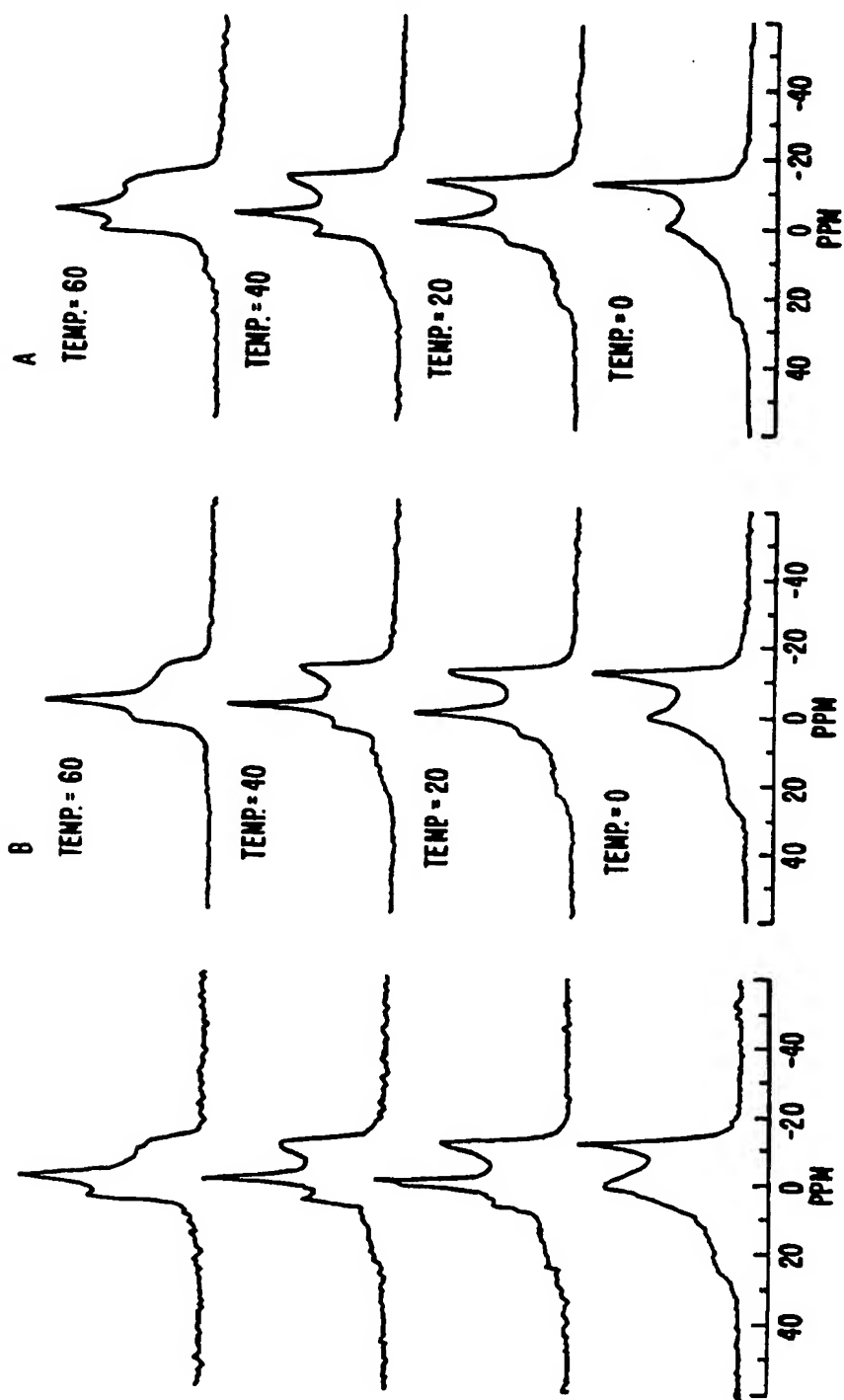
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**FIG. 2.****SUBSTITUTE SHEET**

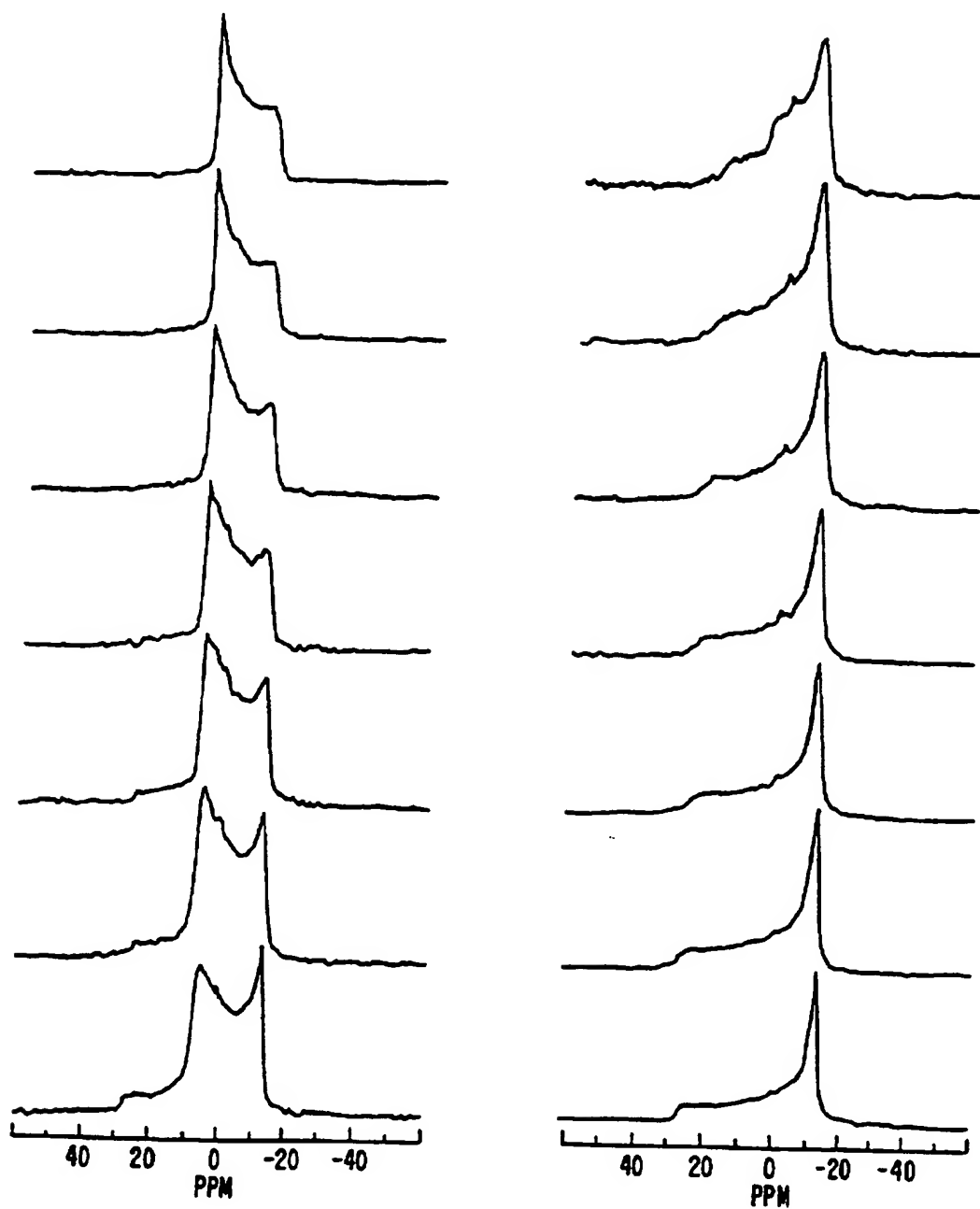
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**FIG. 3.****SUBSTITUTE SHEET**

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**FIG. 4.****SUBSTITUTE SHEET**

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**FIG. 5.****SUBSTITUTE SHEET**

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FIG. 6.

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FIG. 7.

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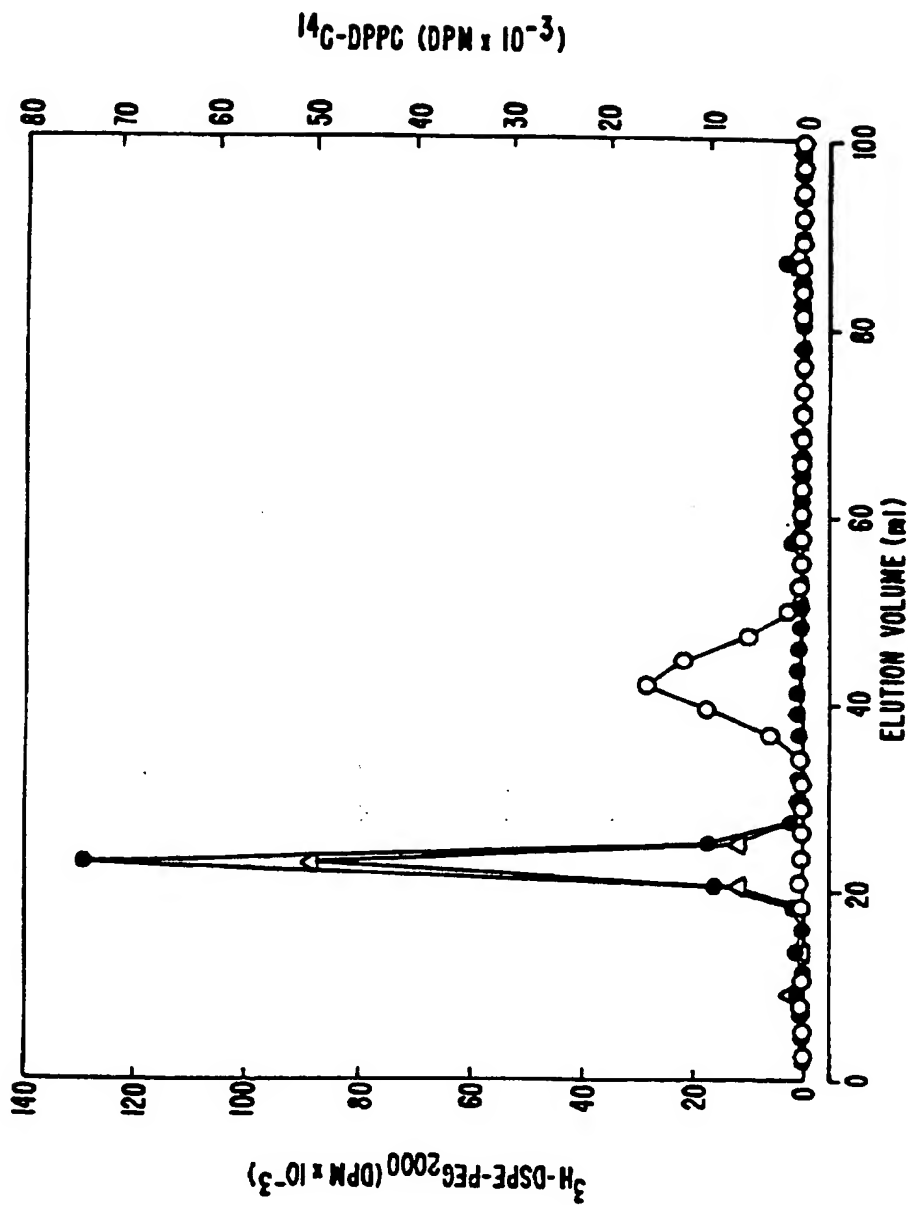
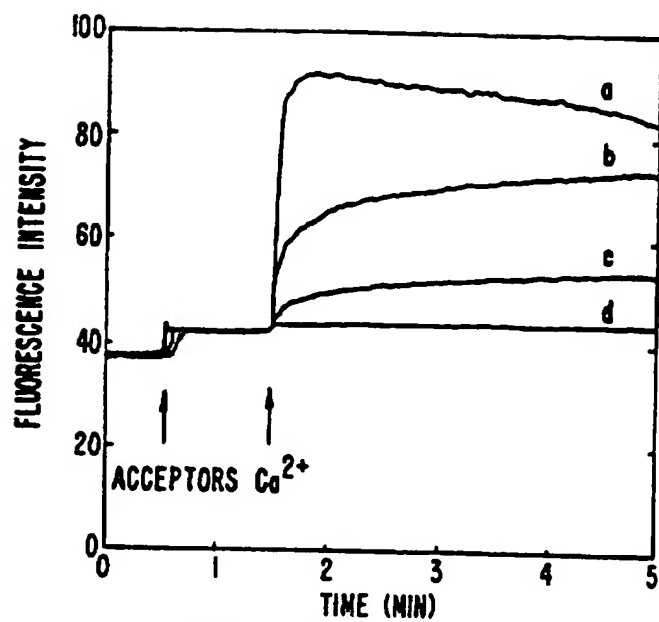
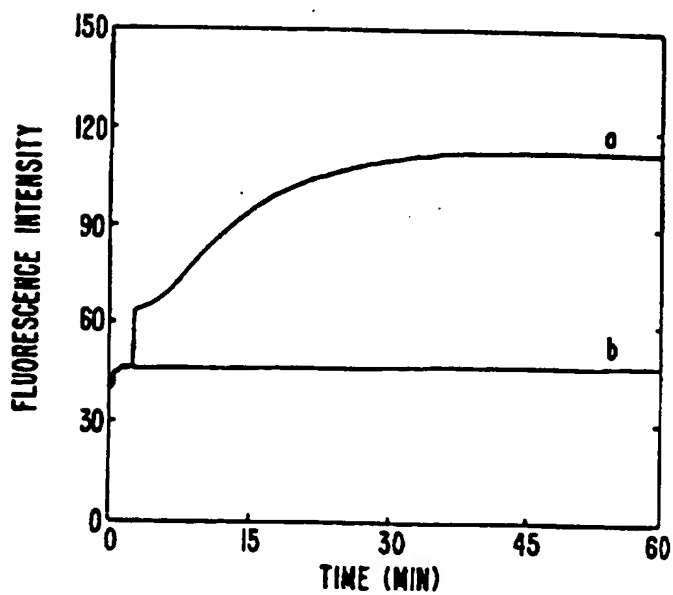
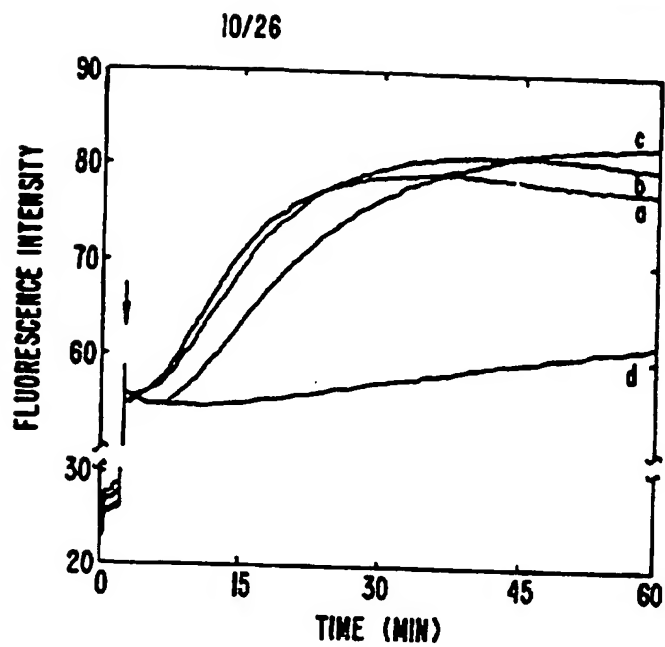
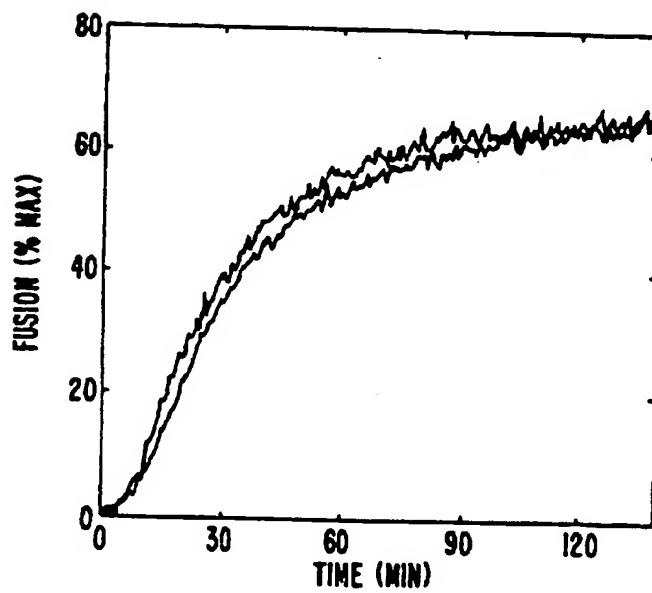


FIG. 8.

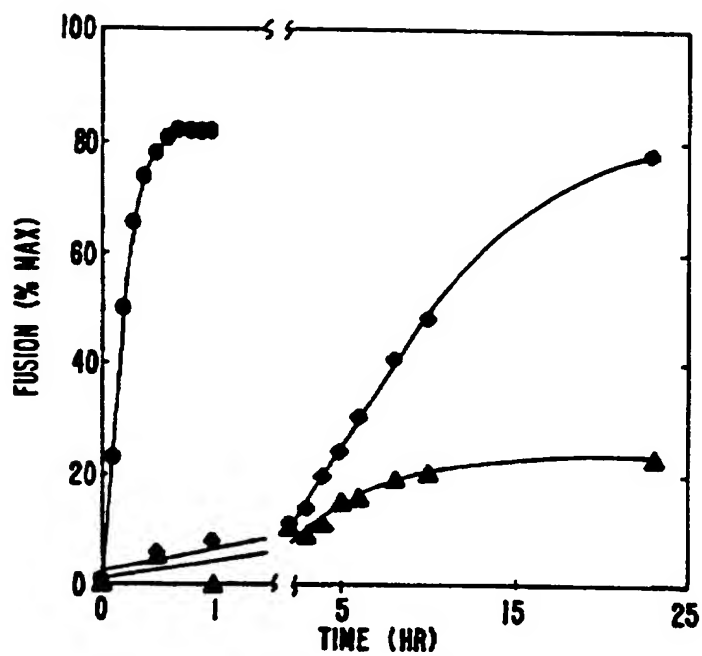
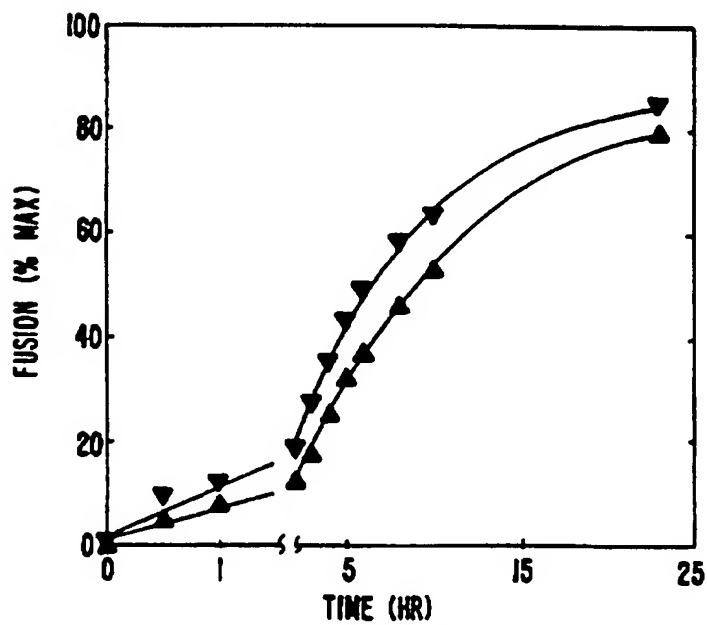
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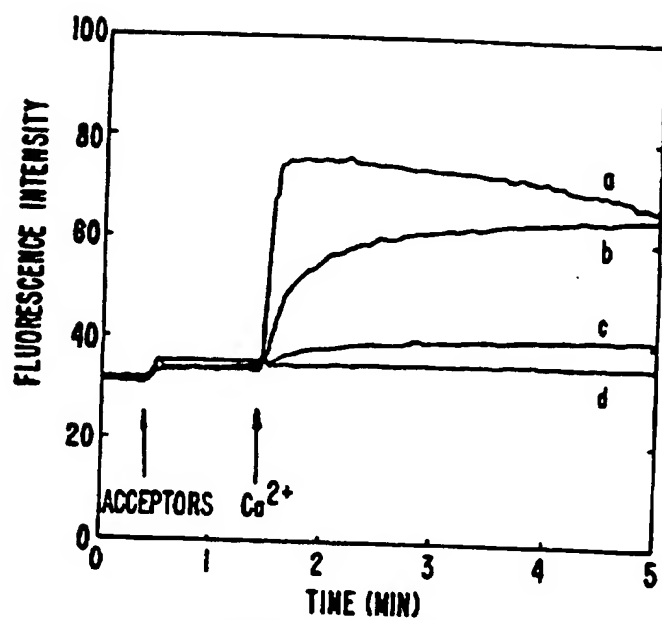
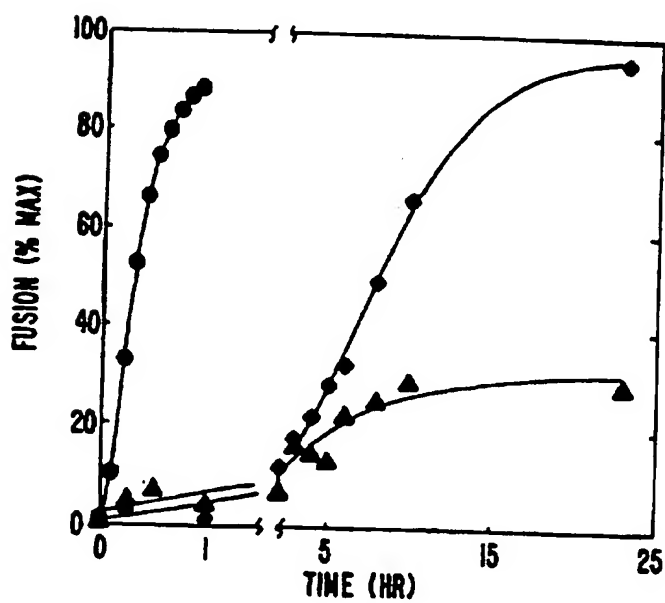
**FIG. 9.****FIG. 10.****SUBSTITUTE SHEET**

**FIG. 11.****FIG. 14.****SUBSTITUTE SHEET**

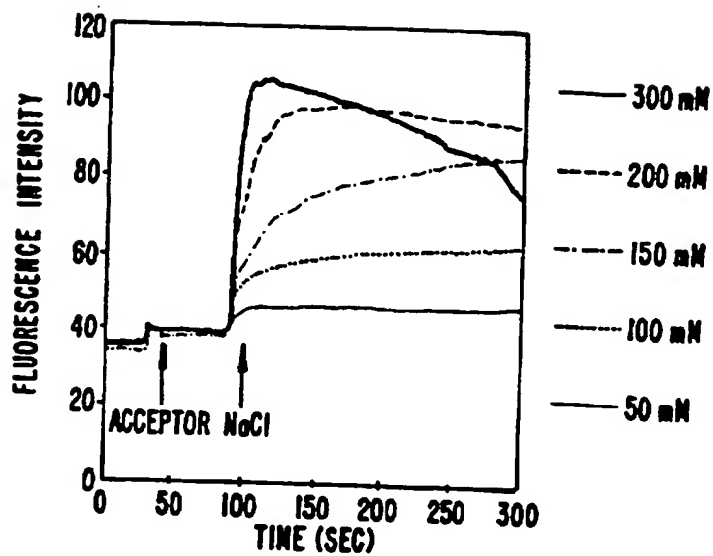
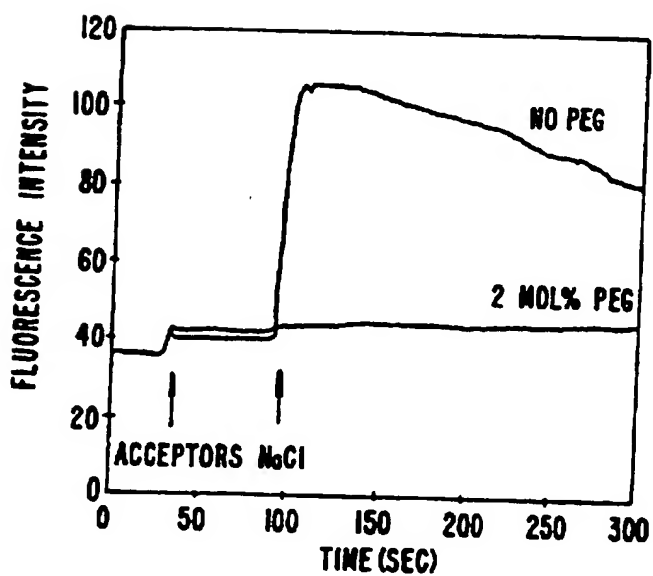
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**FIG. 12A.****FIG. 12B.****SUBSTITUTE SHEET**

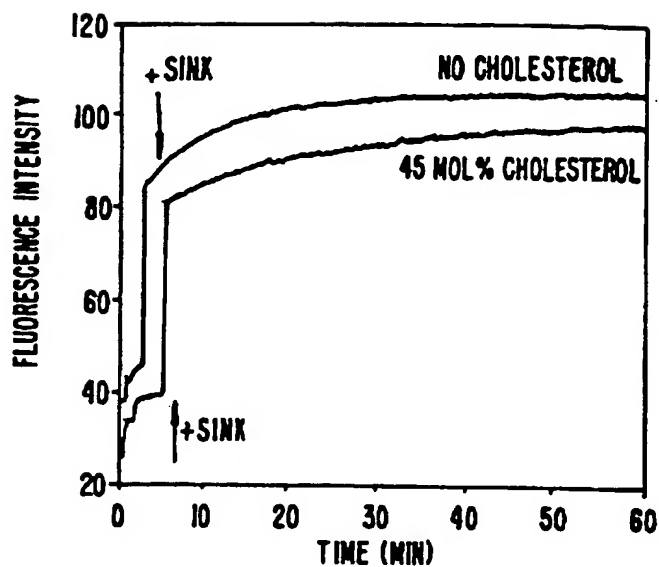
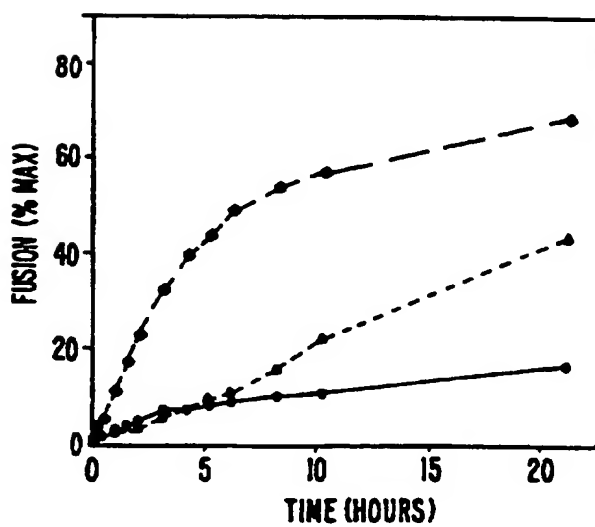
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**FIG. 13A.****FIG. 13B.****SUBSTITUTE SHEET**

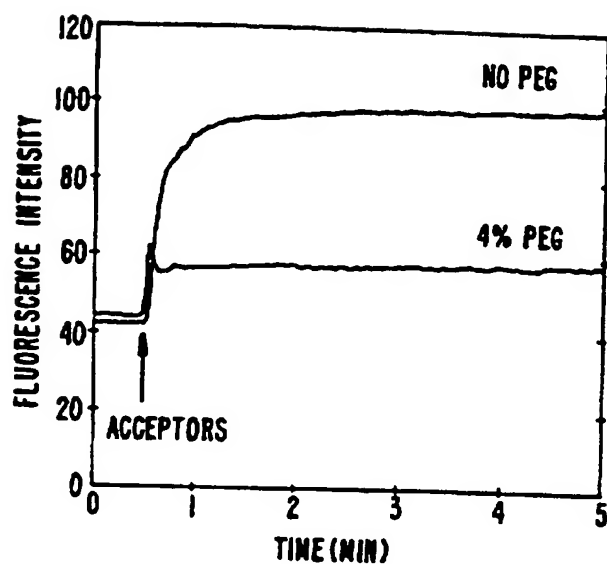
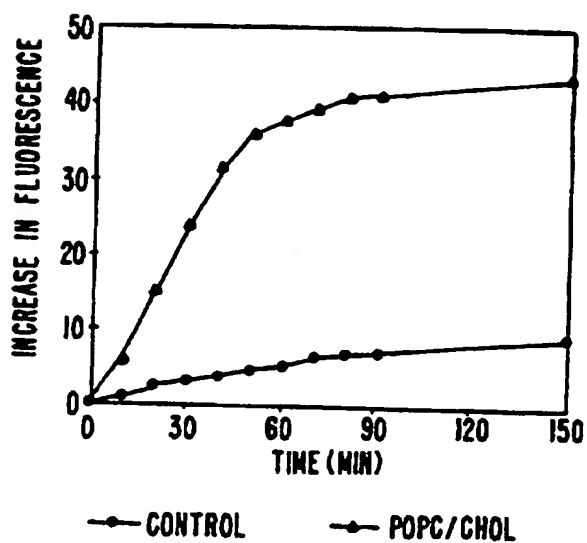
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**FIG. 15.****FIG. 16.****SUBSTITUTE SHEET**

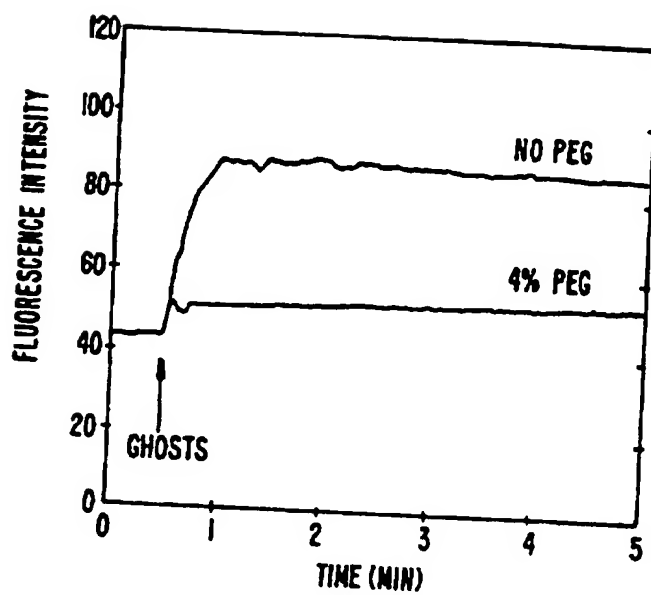
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**FIG. 17.****FIG. 18.****SUBSTITUTE SHEET**

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**FIG. 19.****FIG. 20.****SUBSTITUTE SHEET**

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**FIG. 21.****SUBSTITUTE SHEET**

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FIG. 22A.

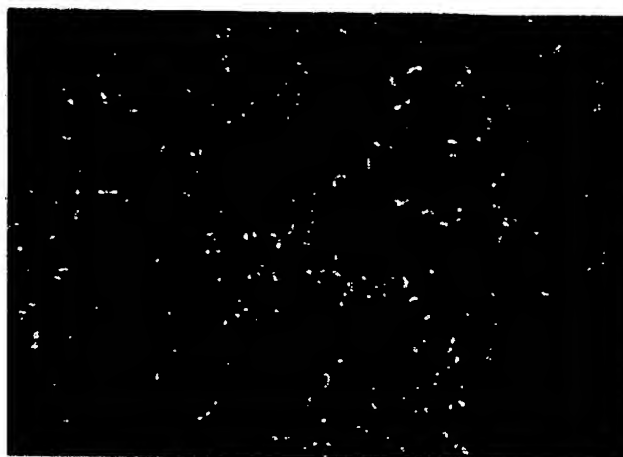
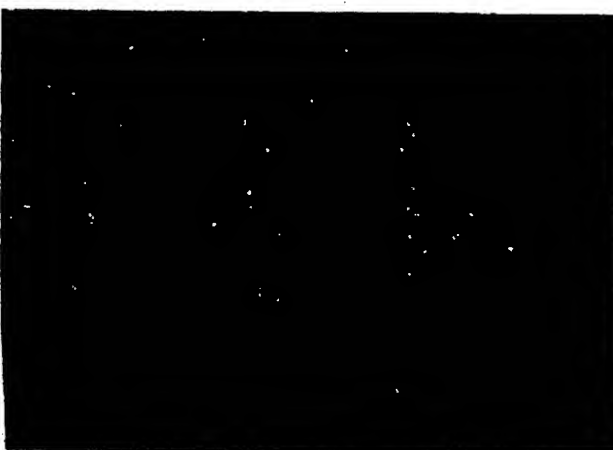


FIG. 22B.



FIG. 22C.



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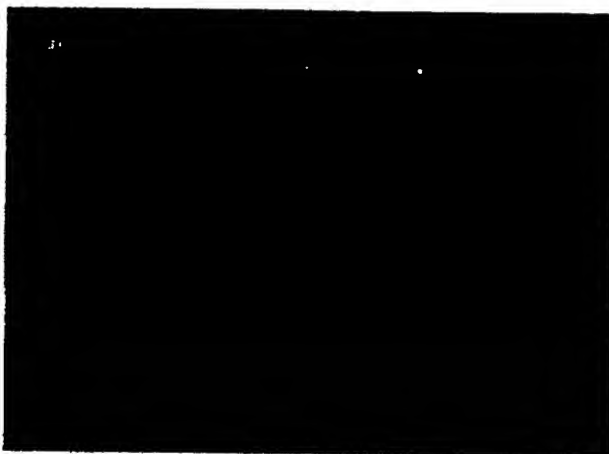


FIG. 22D.



FIG. 22E.

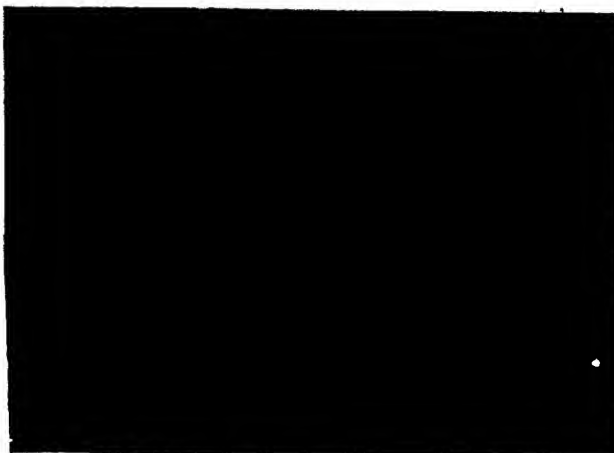


FIG. 22F.

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FIG. 23A.

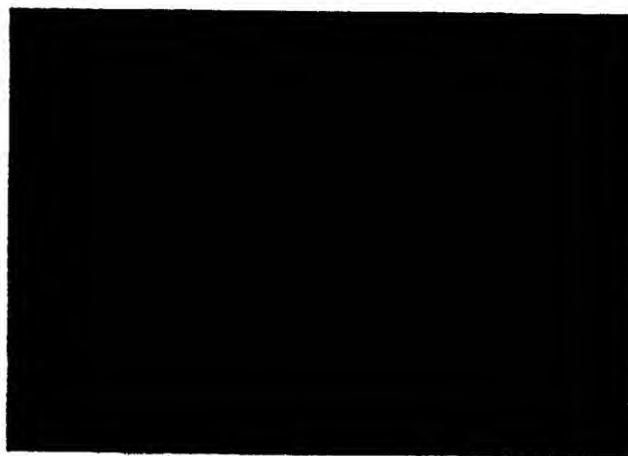


FIG. 23B.



FIG. 23C.

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FIG. 23D.



FIG. 23E.

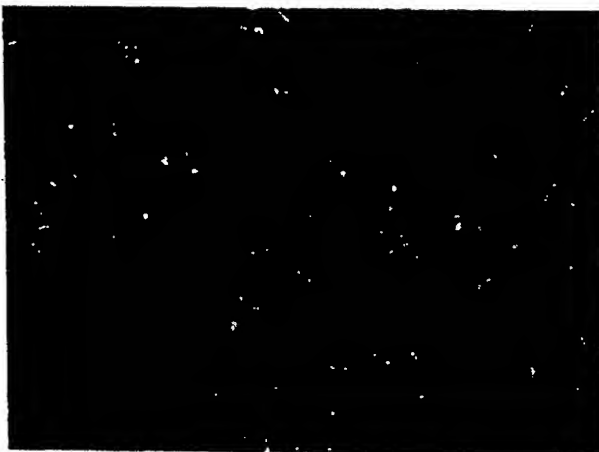
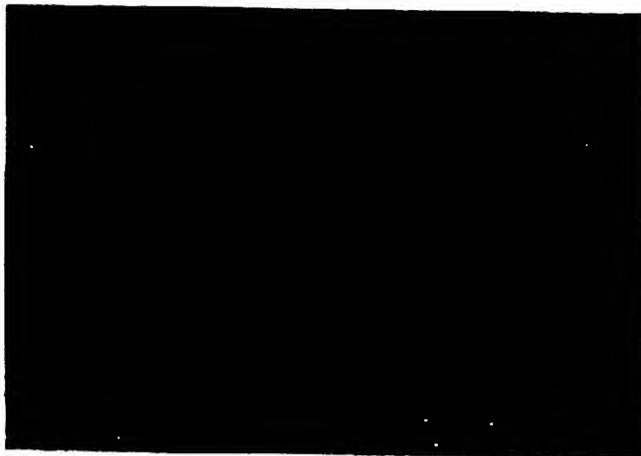


FIG. 23F.



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FIG. 24A.

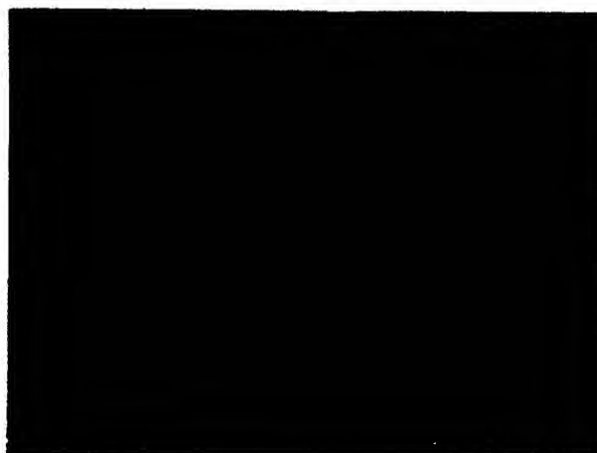


FIG. 24B.



FIG. 24C.

SUBSTITUTE SHEET

FIG. 24D.

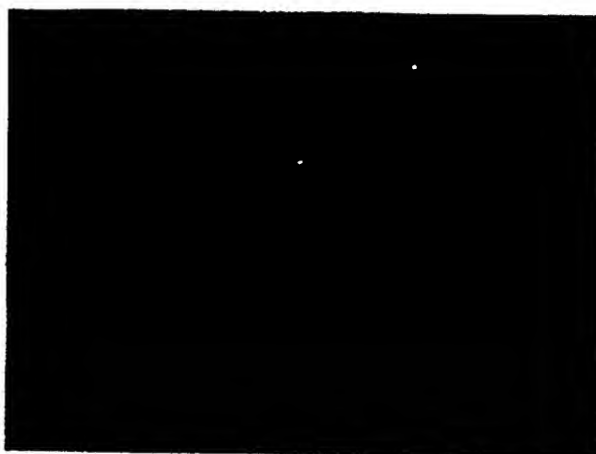
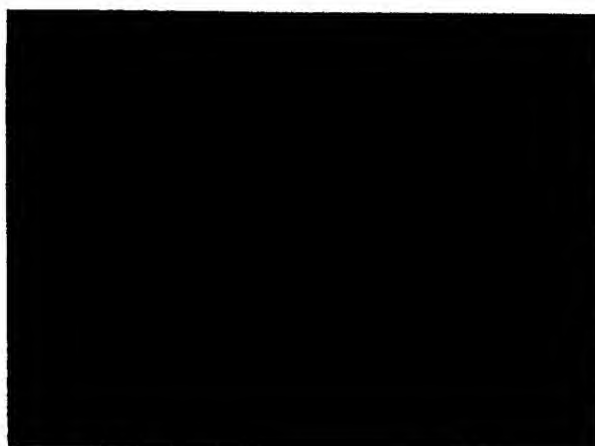


FIG. 24E.

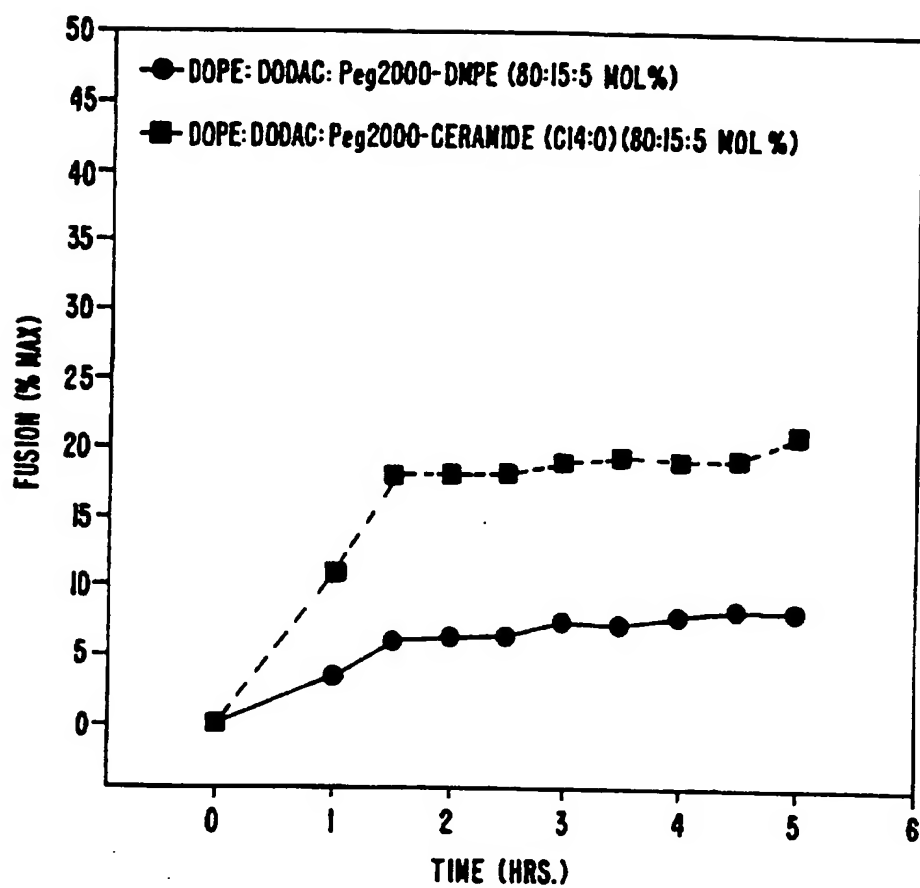


FIG. 24F.

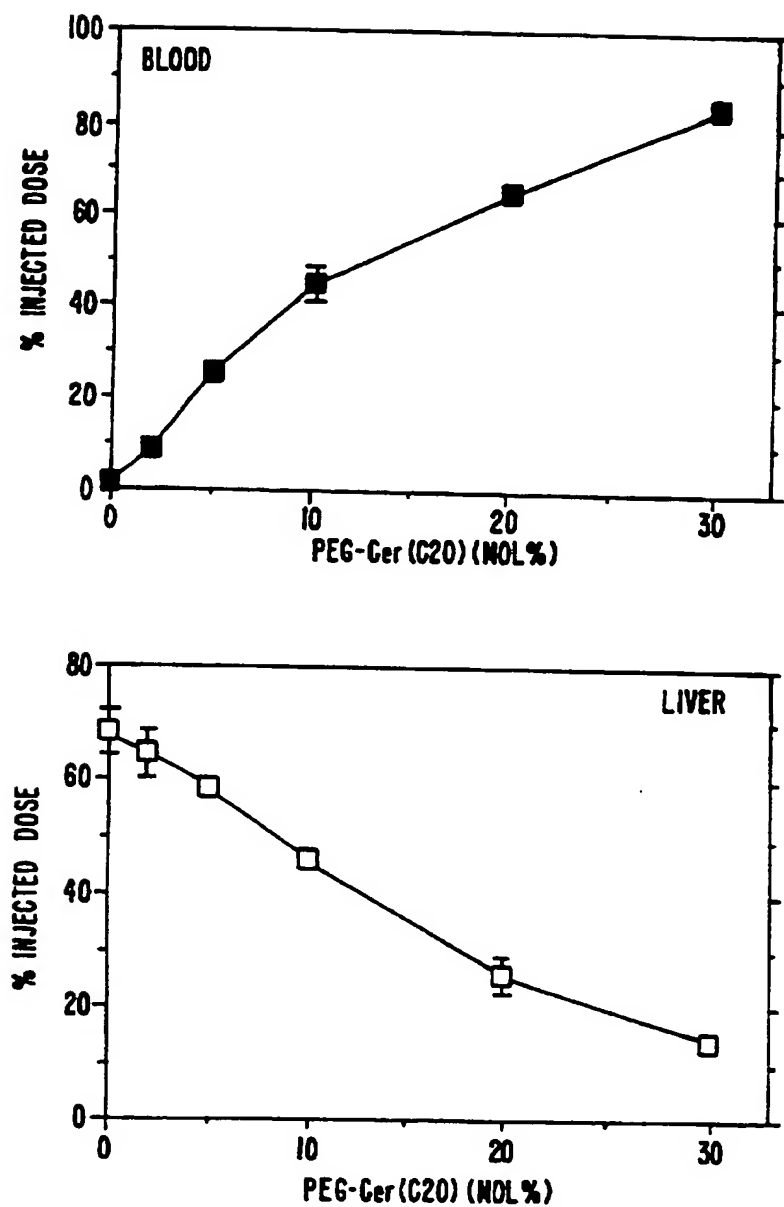


SUBSTITUTE SHEET

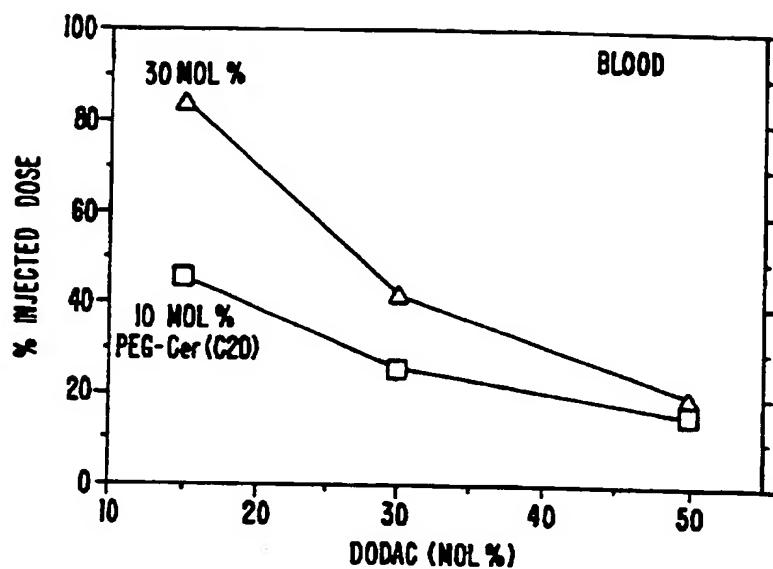
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**FIG. 25.**

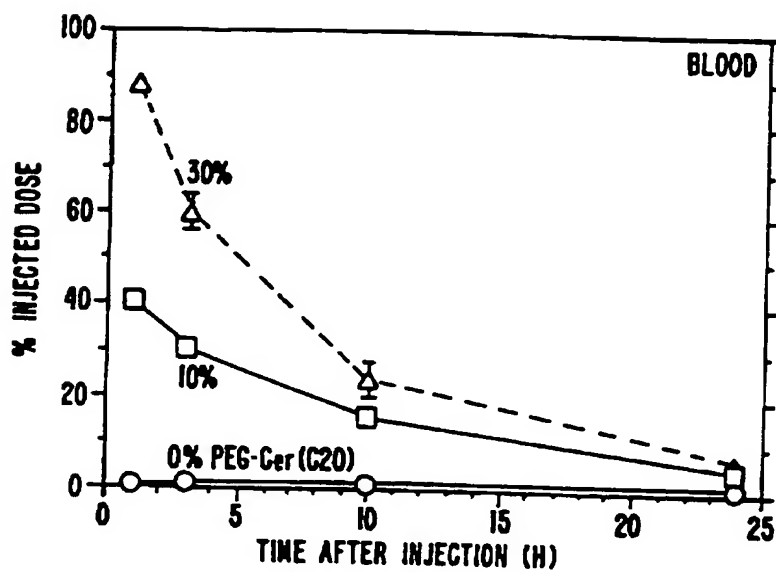
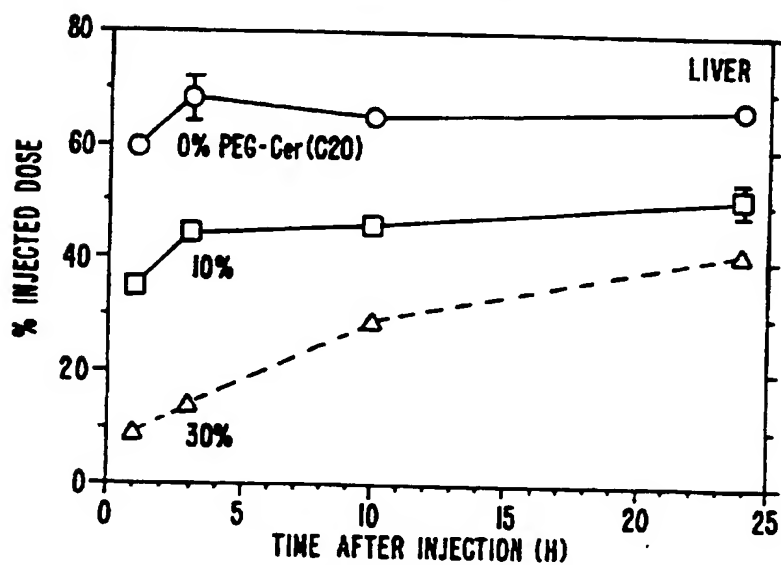
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**FIG. 26.****SUBSTITUTE SHEET**

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**FIG. 27.****SUBSTITUTE SHEET**

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**FIG. 28A.****FIG. 28B.****SUBSTITUTE SHEET**

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/CA 95/00557

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K9/127

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF LIPOSOME RESEARCH, vol. 2, no. 3, 1992 NEW YORK (US), pages 321-334, XP 000303896 A.L. KLIBANOV ET AL. 'LONG-CIRCULATING LIPOSOMES: DEVELOPMENT AND PERSPECTIVES' see the whole document	1-3, 8-13, 15-25, 27, 31-38,40
Y	see page 328, line 27 - line 35	4-7, 28-30, 41-43
Y	--- JOURNAL OF LIPOSOME RESEARCH, vol. 3, no. 1, 1993 NEW YORK (US), pages 17-30, XP 000349732 XIANG GAO ET AL. 'CATIONIC LIPOSOMES AND POLYMERS FOR GENE TRANSFER' see the whole document --- -/-	5, 6, 29, 42

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

20 December 1995

Date of mailing of the international search report

16.01.96

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Benz, K

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INTERNATIONAL SEARCH REPORT

b. International Application No.

PCT/CA 95/00557

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 422 543 (THE GREEN CROSS CORPORATION) 17 April 1991 see the whole document ---	4,7,28, 30,41,43
X	JOURNAL OF LIPOSOME RESEARCH, vol. 4, no. 1, March 1994 NEW YORK (US), pages 361-395, XP 000450216 CHUN-JUNG CHU ET AL. 'PH-SENSITIVE LIPOSOMES' see the whole document -----	1-3,7,8, 15-24, 30-37, 40,43

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA95/00557

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 23-35 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

Information on parent family members

PCT/CA 95/00557

Form PCT/13A/268 (patent family annex) (July 1992)